The role of transcription factors in eosinophil differentiation

De rol van transcriptie factoren in eosinofiel differentiatie

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht
Op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
dinsdag 24 september 2002 des middags om 12.45

door

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Geboren op 12 juli 1973, te Arnhem

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Print: FEBODRUK BV, Enschede

The research described in this thesis was performed at the Department of Pulmonary Diseases, University Medical Center, Utrecht, The Netherlands and was financially supported by a grant from GlaxoSmithKline

Publication of this thesis was financially supported by

J.E. Jurriaanse Stichting
Universiteit Utrecht

ISBN nummer: 90-393-3101-4

Voor Erwin

Voor mijn vader en moeder

Contents

List of Abbreviations

4-OHT	4-hydroxytamoxifen
AP-1	activator protein 1
BA	butyric acid
βc	common beta chain
C/EBP	CCAAT/enhancer binding protein
cAMP	cyclic AMP
CAT	chloramphenicol acetyltransferase
CFU	colony forming unit
CMP	common myeloid progenitor
CREB	cAMP responsive element binding protein
CREM	cAMP responsive element modulator
DN-C/EBP	dominant negative C/EBP
ECP	eosinophil cationic protein
EDN	eosinophil derived neurotoxin
eGFP	enhanced green fluorescent protein
EPO	eosinophil peroxidase
ER	estrogen receptor
FOG	friend of GATA
G-CSF	granulocyte colony stimulating factor
G-CSF-R	G-CSF receptor
GM-CSF	granulocyte macrophage colony stimulating factor
GM-CSF-R	GM-CSF receptor
GM-CSF-Rα	GM-CSFreceptor alpha chain
HSC	hematopoietic stem cell
IL	interleukin
IL-3	interleukin-3
IL-5	interleukin-5
IL-5R	IL-5 receptor
IL-5Rα	IL-5 receptor alpha chain
IRES	internal ribosome entry site
MBP	major basic protein
M-CSF	macrophage colony stimulating factor
M-CSF-R	M-CSF receptor
STAT	signal transducer and activator of transcription

Chapter 1

General introduction

Contents

Eosinophils

Eosinophils originate from myeloid stem cells, develop in the bone marrow, remain for a short period of time in the peripheral blood (6-8h) and then migrate into the tissue [1; 2]. The number of eosinophils in the blood of healthy individuals is normally quite low, about 1-3% of total leukocytes. Paul Ehrlich first recognized the eosinophil in 1879 as cells that contain many granules that stain bright red with the acidic dye eosin [3]. The cytoplasmic granules that are stained by eosin are the main distinguishing feature of eosinophils. These granules contain numerous cytotoxic proteins as well as pro- and anti-inflammatory mediators. Another characteristic of eosinophils is their bilobed nucleus. While eosinophils play a beneficial role in helminth infection, they can also cause tissue damage at inflammatory loci as seen in allergic asthma.

Priming or pre-activation of eosinophils by cytokines and chemokines is critical for optimal activation [4]. Priming by itself does not induce effector responses of eosinophils but instead elevates the activation status of the cell. Upon addition of a second stimulus, the cellular responses are much stronger than those induced by the stimulus alone. For example, eosinophils primed with interleukin 3 (IL-3), IL-5 or granulocyte-macrophage colony stimulating factor (GM-CSF) have an enhanced respiratory burst in response to opsonized particles and migrate better in response to platelet activating factor [5; 6].

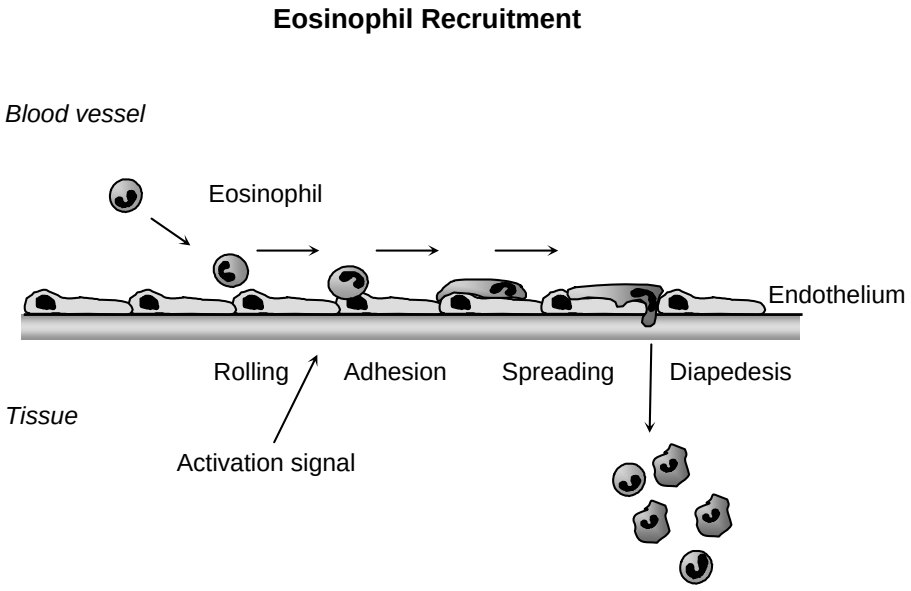

Figure 1. Multi step model for eosinophil extravasation. Cells roll on activated endothelial cells. Activation results in firm adhesion interactions with ligands present on the endothelium. Finally, the cells spread and migrate across the endothelium cells to the site of inflammation.

Eosinophils generally constitute only a small proportion of the total leukocyte number in the blood. Concentration gradients of chemotactins and expression of the correct adhesion molecules guide the cell out of the bloodstream and into the tissues. The unique combination of these mediators results in the selective recruitment of eosinophils. Eosinophil recruitment is a multi-step process, including rolling along and attachment to endothelial cells, trans-endothelial diapedesis, and migration into the tissue (figure 1) [7; 8]. Rolling of the eosinophils along the endothelial cells is the first step in the extravasation process and this reversible adhesion is mainly mediated by selectins [9]. Three types of selectins are known, L(eukocyte)-, E(ndothelium)-, and P(latelet)-selectin [9]. L-selectin is constitutively expressed on leukocytes, whereas P-selectin is expressed on activated platelets. P- and E-selectin are expressed on endothelial cells activated by pro-inflammatory cytokines such as IL-1 and tumor necrosis factor [10]. The selectins attach to a specific glycoproteins, such as PSGL-1 (P-Selectin Glycoprotein Ligand-1), ESL-1 (E-Selectin Ligand-1), and MadCAM (Mucosal Addressin Cell Adhesion Molecule-1) [11; 12; 13; 14]. Activation of the rolling cell by chemo-attractants induces firm adhesion and subsequent spreading of the cell, which are mediated by integrins [15]. Integrins are cell-surface receptors responsible for cell attachment to extracellular matrices and to other cells, including endothelial cells, by binding to their counter ligands, which belong to the immunoglobulin superfamily [16]. Next, the cell migrates to the site where endothelial cells connect to each other and passes between them to the tissue [17]. When cells have passed the endothelium, they interact with the extracellular matrix proteins such as fibronectin.

Once eosinophils are present in the tissue, activation can result in cytotoxicity. Eosinophils utilise two main cytotoxic mechanisms: degranulation and the respiratory burst (figure 2). Degranulation occurs through the secretion of granule-associated toxic cationic proteins, enzymes and lipid mediators. Three types of granules are recognized at different stages of eosinophilic maturation. The primary (coreless) granules are round, uniform and observed at the stage of eosinophilic promyelocyte in the bone marrow [18]. As eosinophils differentiate, the primary granules develop into core-containing specific granules [18]. Eosinophil Peroxidase (EPO), Major Basic Protein (MBP), Eosinophil Cationic Protein (ECP), and Eosinophil-Derived Neurotoxin (EDN) are present in both primary and specific granules, but their distribution differs [19]. In primary granules, MBP has a uniform distribution, whereas in specific granules MBP has a crystalloid core distribution, while the matrix surrounding it contains ECP, EPO, and EDN. Exocytosis and piecemeal degranulation represent two functionally different processes by which eosinophils release their granule mediators. Piecemeal degranulation provides the possibility of a long-lasting and selective release of granule content [20; 21]. Eosinophil exocytosis, on the other hand, occurs classically as a regulated event in the context of granule-to-granule or granule-to-plasma membrane fusion and extrusion of nonmembrane-bound granule [22]. A third type of granule, the lipid body, is a non–membrane-bound, lipid-rich cytoplasmic inclusion. Lipid bodies increase in numbers and size in cells associated with inflammation. They would serve as sites at

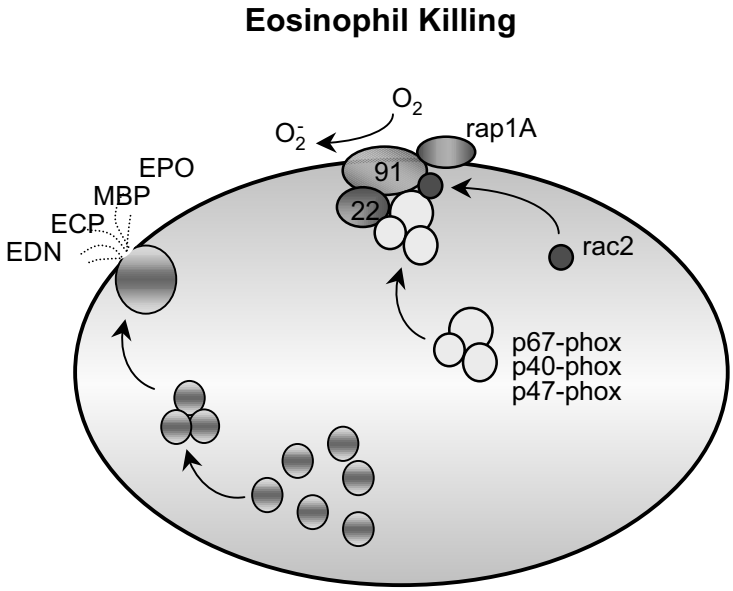

Figure 2. Eosinophil mediated killing. Eosinophils utilize two cytotoxic mechanisms, degranulation and the respiratory burst. Degranulation occurs through the secretion of granule-associated toxic cationic proteins, enzymes and lipid mediators. During the respiratory burst, the eosinophil produces toxic oxygen metabolites. These reactive oxygen species (ROS) are produced by a membrane-bound NADPH-oxidase, which reduces molecular oxygen into superoxide. These two mechanisms synergize in the killing reaction

which the coordinated and regulated enzymatic events involved in arachidonate mobilization and oxidative metabolism could occur [23].

During the respiratory burst, the eosinophil produces toxic oxygen metabolites. These reactive oxygen species (ROS) are produced by a membrane-bound NADPH-oxidase, which reduces molecular oxygen into superoxide. Subsequently, this superoxide can be further converted into other ROS species, for example hydrogen peroxide. These oxygen metabolites are cytotoxic for micro-organisms and synergize with toxic granule proteins (e.g. EPO) to facilitate microbial killing [24; 25].

Apart from being important effector cells, eosinophils can act as pro-inflammatory cells. They have the capacity of producing large amounts of cytokines and chemokines including IL-4, IL-5, GM-CSF, interferon-γ, TNF-α, RANTES (regulated on activation, normal T-cell expressed and secreted), IL-8, and IL-10 [26-30]. In addition, several bioactive lipids are formed for example platelet activating factor and leukotrienes C_4 and B_4 [31; 32]. All these mediators are involved in various stages of the inflammatory response and can act on eosinophils or other inflammatory cells, contributing to ongoing inflammation. Furthermore, eosinophils are reported to have anti-inflammatory properties. They have the ability to release enzymes such as histaminase and arylsulfatase, which are recognised to degrade histamine and leukotrienes respectively [33; 34].

The role of eosinophils in diseases

Eosinophils are strongly associated with the defence against infections caused by macro parasites, especially helminths [35; 36]. Helminth infections cause an increase in the number of circulating eosinophils and a massive eosinophil infiltration can be observed at the site of the parasite [37; 38]. Eosinophils can bind to the surface of the helminths *in vitro*, both larval and adult forms, thereby damaging the surface of the helminth by release of granule proteins such as MBP and ECP or by peroxidation via EPO [39; 40]. Oxidative reactions are not essential for the killing, but they can clearly enhance the toxic effect of the cationic proteins.

In contrast to this beneficial role in host defence, eosinophils are also thought to play an important role in the pathogenesis of allergic diseases, such as allergic asthma [41; 42]. Allergic asthma is a complex clinical syndrome characterised by variable airflow obstruction, bronchial hyper-responsiveness and chronic airway inflammation [43; 44]. The acute allergic reaction usually occurs within minutes of antigenic challenge and is generally referred to as the early-asthmatic response (EAR). The EAR is followed 2 to 6 hours later by the late-asthmatic response (LAR), which involves infiltration of the airways by eosinophils, mononuclear cells, and lymphocytes as well as elaboration of T_H2 cytokines and chemokines [45; 46]. The T_H2 cytokines IL-4 and IL-13 regulate IgE expression by B-lymphocytes, whereas tumor necrosis factor α, IL-1, and chemokines (e.g., IL-8) are responsible for cellular recruitment. IgE secreted by B-lymphocytes binds to high-affinity IgE receptors on human mast cells and basophils [47-50]. Interaction of allergen with the specific IgE molecules on the surface of these cells causes cross-linking and triggering of the cell receptor with concomitant activation of mast cells and basophils. Together with chronic signals by the innate immune system the inflammation persists. The level of eosinophilic inflammation of the airway in asthmatics correlates with the severity of asthma [43; 51]. Eosinophils are believed to cause long-term tissue damage when activated to release their granule content of cationic proteins, including EPO, EDN, MBP, and ECP. The capacity of these proteins to damage airways epithelium has been demonstrated *in vitro* and *in vivo* [52-55]. In response to allergic stimuli in the lung, the eosinophil pool in the bone marrow expands and the number of eosinophils residing in the blood and at the site of allergen provocation increases markedly [41; 42].

Hematopoiesis

The human hematopoietic system of produces about one trillion blood cells each day [56]. This continuous process involves the sequential commitment of multipotential hematopoietic stem cells (HSC) to gradually more-restricted progenitor cells, and finally to the functionally distinct cells found in peripheral blood: erythrocytes, platelets, neutrophils, eosinophils, monocyte/ macrophages, and lymphocytes. Within the first two stages extensive expansion/proliferation of cells occur. Hematopoiesis occurs at distinct anatomic sites during human development [57-

[59]. Embryonic hematopoiesis begins when blood develops from the mesoderm in the yolk sac. Definitive hematopoiesis is initiated in the fetal liver, and occurs in the bone marrow after birth.
HSC are currently defined as single cells that are capable of self-renewal. These cells can also differentiate into a defined set of committed progenitors [60; 61]. They are negative for markers found on T cells, B cells, granulocytes, mono/macrophages, erythrocytes, NK cells etc [62]. These stem cells are very rare, about only one in 10,000 bone marrow cells. Human HSC have been isolated from umbilical cord blood, fetal liver, fetal and adult bone marrow, and mobilized peripheral blood (MPB) [63; 64]. HSC can be divided into long-term repopulating (LT-HSC) and short-term repopulating cells (ST-HSC) based on degree of self-renewal [65]. Long-term self-renewal is limited to LT-HSC in the hematopoietic stem and progenitor pool [66]. Despite intensive investigation, the mechanism underlying the genetic control of self-renewal and LT-HSC expansion remains undefined. All leukemias and clonogenic cancer cells have gained (or retained) the properties of self-renewal, therefore it is crucial to define the genetic control of the LT-HSC class of stem cells.
As shown in figure 3, downstream of the clonogenic multipotent pool are two developmental choices, either myeloid or lymphoid [61; 67; 68]. This raises a question: what are the molecular mechanisms that dictate the fate of a common precursor to a particular lineage? Over the years, several models have been proposed that describe the hematopoietic lineage determination.
The inductive model suggests active roles for instructive signals in directing stem cells to commit to a particular hematopoietic lineage, such as signals provided by lineage-specific cytokines or stromal cells [69; 70]. This is supported by findings that cytokines such as erythropoietin and thrombopoietin provoke differentiation by inducing specific factors, such as signal transducers and activators of transcription (STAT) or cyclin dependent kinase inhibitors [71-76].
On the other hand the stochastic model describes mechanisms in the pluripotent cell that determine their hematopoietic fate and are independent of extracellular signals [77]. The role of cytokines is merely to provide survival and proliferation signals. This is supported by recent work which has shown that introduction of the anti-apoptotic bcl-2 gene into a pluripotent hematopoietic cell line, results in multi-lineage differentiation of these cells in the absence of cytokines [78]. It is also possible that a combination of these two models exist. Stochastic processes determine the preferred differentiation patterns, but exposure to a particular cytokine pathway may re-enforce or override the selection.

Granulopoiesis

The development of mature hematopoietic cells from the pluripotent HSC involves a regulated progression through lineage commitment, terminal differentiation, and growth arrest. The first two lineage progenitors recognized are the common lymphoid progenitor and the common myeloid progenitor or granulocyte/erythroid/macrophage/megakaryocyte colony-forming unit (GEMM-CFU). Each of these progenitors is a clonal precursor of a limited subset of progeny, and has a distinct expressed gene profile [61; 79; 80].

GEMM-CFU can give rise to more lineage-restricted progenitors: the megakaryocyte/erythrocyte CFU, the granulocyte/monocyte CFU, and the eosinophil/basophil CFU [81; 82]. The presence of a common late stage progenitor of eosinophils and basophils was demonstrated by a common progenitor in clonal assays, shared content of the major granule proteins and proteoglycans and the occurrence of various forms of transition between the two cells types. Under normal conditions the clonogenic precursors, the megakaryocyte/erythrocyte CFU, the granulocyte/ monocyte CFU, and the eosinophil/basophil CFU are absolutely lineage restricted and do not self-renew upon transplantation [83]. These cells are capable to differentiate via their various precursor cells into mature blood cells (figure 3).

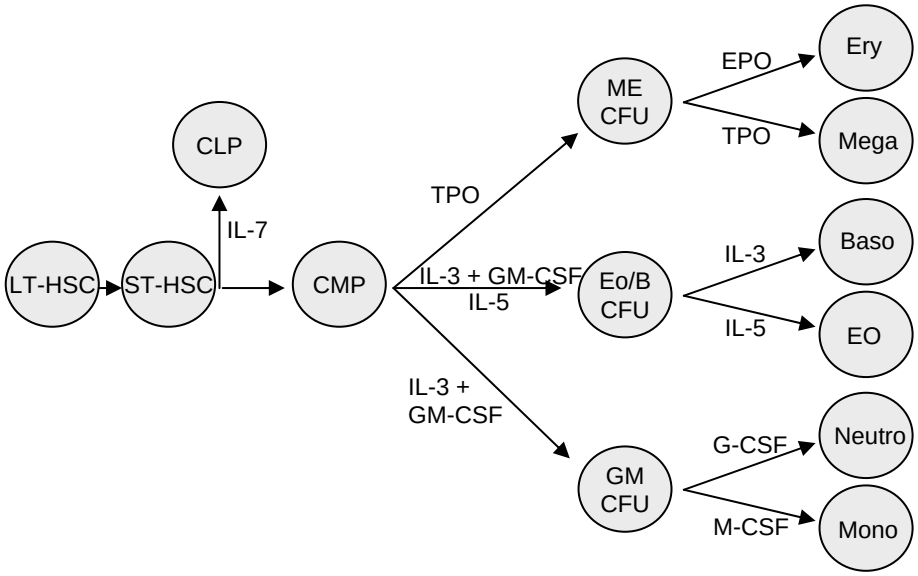

Figure 3. A model for hematopoiesis. Linear model for the differentiation from hematopoietic stem cell into the different mature cells, the lineage specific cytokines are added to the different branches. CLP: common lymphoid progenitor. CMP: common myeloid progenitor. ME-CFU: megakaryocyte and erythrocyte progenitor. Eo/B-CFU: eosinophil and basphil progenitor. GM-CFU: granulocyte and monocyte progenitor. Mega: megakaryocyte. Ery: erythrocyte. Baso: basophil. EO: eosinophil. Neutro: neutrophil. Mono: monocyte.

Cytokines in hematopoiesis

The inductive model describes active roles for lineage-specific cytokines in directing stem cells to commit to a particular hematopoietic lineage (figure 3). Cytokines are pleiotropic; they can act on different cells and induce a wide range of responses. These multiple biological effects can be explained by the range of signalling components and transcription factors present in the target cells at the time of stimulation with the cytokine. However, several cytokines can act on one target cell to elicit the same response. This can be explained, in part, by the fact that the receptors for cytokines share common subunits important for signal transduction [84; 85].

Interleukin-7 (IL-7) is a major factor in stimulating bone marrow stem cells to differentiate towards lymphocytes (mostly B cells and T cells). The hormone thrombopoietin, secreted by the liver, induces the lineage leading to megakaryocytes and erythrocytes. Erythropoietin induces the production and differentiation of erythrocytes. The production of megakaryocytes and their fragmentation into platelets is stimulated by thrombopoietin assisted by Interleukin-11. Interleukin-3 (IL-3) participates in the differentiation of most of the white blood cell lineages but plays a particularly prominent role in the formation of basophils. Granulocyte-macrophage colony-stimulating factor (GM-CSF), as its name suggests, directs cells down the lineage leading to both those cell types. Further differentiation into neutrophils is under the influence of granulocyte colony-stimulating factor. On the other hand, stimulation of the granulocyte/ macrophage progenitor cells by macrophage colony-stimulating factor induces differentiation into monocytes, the precursors of macrophages.

Certain cytokines appear to be more restricted in their actions than others, both in terms of lineage and the stage of differentiation of the responsive cells. Some cytokines promote the growth and survival of progenitors and support the development of more than one lineage of cells, like IL-3 and GM-CSF. In contrast, other cytokines act primarily on a particular lineage of cells, such as granulocyte colony-stimulating factor and erythropoietin.

Cytokine receptors

Cytokines regulate their function through specific receptors. Most cytokine receptors consist of multiple subunits, one of which is cytokine specific. The cytokine specific subunits are involved in the specific binding of ligand, while the common components mediate cytokine activated signal transduction pathways and can be shared between several cytokine receptors. The receptors for IL-2, IL-4, IL-9, and IL-15 all use the same signal transducing γ chain [86; 87; 88]. Therefore, these cytokines generally activate similar signal transduction pathways in various cell types.

The receptors for IL-3, IL-5, and GM-CSF each consist of two distinct membrane proteins. The α-chain is cytokine-specific and binds the ligand with low affinity, and the common β chain (βc) is shared among the receptors [89-93]. Although the βc-chain does not bind IL-5 by itself, it forms a high affinity receptor by dimerisation with the β-chain [91; 94]. Upon dimerisation, multiple tyrosine

residues in the βc become phosphorylated, mainly by members of the Janus kinase (JAK) family [95; 96]. The phosphorylated receptor provides docking sites for a variety of Src-homology 2 (SH2) domain-containing proteins, including STATs (signal transducers and activators of transcription). In the last decade many of the signal transduction pathways regulated by the phosphorylated βc have been identified, including the PI-3K/PKB and the Ras-ERK pathways [95].

Although the βc initiates signal transduction events specific for this cytokine family, it will not distinguish between IL-3, IL-5, and GM-CSF. The cytokine specific α-chains on the other hand are likely candidates for mediating specificity in signalling between cytokines of one family. Although the intracellular domains of the α-chains are rather short, they play a role in signal transduction, since deletion of the cytoplasmic domains abrogates receptor signalling [97]. Recently mechanisms of transcriptional activation by cytokine-specific receptor subunits were identified. Syntenin associates with the cytoplasmic tail of the IL-5Rα. Syntenin was found to directly associate with the transcription factor Sox4. Association of syntenin with IL-5Rα was required for IL-5-mediated activation of Sox4 [98]. Furthermore, a novel protein GRAP (GM-CSF receptor α subunit-associated protein) was identified which binds to GM-CSFα, indicating that it may play a role in GM-CSF specific signal transduction [99].

Expression of the cytokine specific β-chain may be critical to the entry of multipotential myeloid progenitors into a particular hematopoietic developmental program.

IL-5 in eosinophil differentiation

Interleukin-5 is produced in lymphocytes, mast cells, eosinophils, and airway smooth muscle and epithelial cells [100; 101]. Biologically active interleukin-5 is a disulfide-linked homodimer that is held together by the highly conserved cysteine residues that orientates the monomers in an anti parallel arrangement [102; 103; 103]. IL-5 promotes growth and differentiation to mature eosinophils in the bone marrow [104; 105]. Other studies showed that $CD34^+$ cells cultured in the presence of IL-5 were committed to the eosinophil lineage and were capable of forming eosinophil colonies [104; 106]. The role of IL-5 and eosinophils is best studied in relation to allergic asthma. Beside the increased levels of IL-5 in peripheral blood of asthmatic patients, also Eo/B-progenitors are elevated in blood of asthmatics after allergen challenge [107-110].

Transgenic mice, in which IL-5 is constitutively expressed in T-cells, show a profound and lifelong eosinophilia, with large numbers of eosinophils in the blood, spleen and bone marrow [111]. In mice with an inactive IL-5 gene, lung eosinophilia was absent and very little inflammation and lung damage was observed after allergen challenge, unless the system is reconstituted with IL-5 [112]. Furthermore, anti-IL-5 treatment prevents pulmonary eosinophilia and bronchohypere sponsiveness in animal models [113; 114], lasting months after a single injection; these effects are consistent with a systemic; perhaps bone marrow, suppression induced by IL-5. Leckie *et al.* studied the effect of a humanized anti-IL-5 antibody in allergic asthmatics and showed a clear reduction in blood eosinophils [115].

Other factors involved in hematopoiesis

Under normal conditions, hematopoiesis takes place within the bone marrow microenvironment. It has been shown that microenvironment where the stem cells reside play critical roles in the determination of their self-renewal or differentiation choices. HSC and the progenitors interact relatively specifically with cell and extracellular matrix ligands present in the bone marrow [116-118]. These adhesive interactions are responsible for the retention of hematopoietic cells in the marrow. Like hematopoietic cells, hematopoietic cytokines bind to some specific extracellular matrix components, and stromal cells can express certain cytokines on their surface [119; 120; 121]. Selective adhesion of progenitors and cytokines to extracellular matrix components or stromal cells then results in the co-localisation of progenitors at a specific stage of differentiation with a specific array of cytokines, in so-termed niches [122; 123].

There is also mounting evidence that contact interactions per se between progenitors and marrow stromal ligands play an important role in the regulation of the hematopoietic process [124; 125; 126]. Adhesive interactions themselves may serve as growth or survival signals or adhesion itself may modulate cytokine- or growth factor-dependent signals. More than 20 different adhesion receptors have been identified on hematopoietic progenitors [127; 128]. These include members of the integrin family responsible for adhesion to ECM components (fibronectin, collagen, laminin, or thrombospondin) or adhesion ligands expressed by other cells [129-131].

Integrins are cell adhesion molecules that can transmit signals between progenitors and bone marrow stromal cells. They are heterodimeric transmembrane glycoproteins, composed of non-covalently associated α and β subunits [16; 132]. Differentiation of β1 knockout precursors is normal *in vitro*. However, these cells accumulate in the blood *in vivo* [133]. This indicates that β_1 integrins are essential for the adhesion of HSC to the vessel wall, and thereby the homing to the bone marrow, but not for their development. Moreover, β1-integrins and their ligands are ubiquitously expressed and β1-integrins are not sufficient for the rather exclusive progenitor-marrow interactions. Thus, another receptor(s) must be responsible for the specific stem cell-marrow interactions. A candidate is the α_4 subunit, which can associate with β_1 and β_7 integrins and is important for the differentiation of erythroid, myeloid and B cell progenitors [134].

Transcription factors

In the stochastic model default mechanisms in the pluripotent cell determine their hematopoietic fate independently of specific extracellular signals. Transcription factors are a possible intrinsic mechanism by which hematopoietic determination occurs. Transcription factors are sequence-specific DNA-binding proteins with a variety of functions that include the initiation of DNA replication and the control of gene transcription [135]. Transcription factors are classified according to the three-dimensional structure of their DNA-binding motifs. Regulation of transcription factor activity occurs through their binding to DNA sequences, the relative concentration of particular

transcription factors within the cell, post-translational modification (phosphorylation and acetylation) and the presence of co-factors [135].

The pattern of gene expression within a cell is established by cell-type specific transcription factors that mediate the net effect of the variety of proliferation and differentiation signals that impinge on it. Therefore, it is interesting to understand more about the role of transcription factors during differentiation. Lineage-restricted transcription factors provide a valuable starting point in searching for the molecular mechanisms of cell differentiation. Gene-knockout studies in mice have provided clues of the essential roles of transcription factors during discrete stages of hematopoiesis. However, in targeted gene disruption of the mouse it is only possible to investigate the first action of a factor in a cascade. Conditional knockouts and analysis of mice that are deficient in more than one transcription factor will be necessary.

In the last decade it has become clear that the expression of lineage specific genes is regulated by the combinatorial association of transcription factors present in the early and committed progenitors, and in more differentiated cells (For review see [136; 137]). Transcription factors regulating expression of lineage specific genes are not exclusively expressed in that particular lineage. However, the overlapping expression patterns and the combinatorial action of these factors may provide the specificity to regulate transcription of lineage specific genes. Many lineage specific genes have binding sites for multiple transcription factors. There are many examples where transcription factors function in concert to upregulate gene expression. For example, AML-1, its binding partner CBFβ, or C/EBP α each not significantly stimulate the myeloid specific M-CSF receptor promoter activity. However, the simultaneous expression of all three proteins was shown to increase the promoter activity 90-fold [138].

Another level of regulation of differentiation is by graded concentrations of transcription factors. Low PU.1 expression in progenitors specifically induces B-cell development, whereas high PU.1 levels suppress the B-cell fate and instead promote macrophage differentiation [139].

Recently, it became clear that besides these positive ways of activation of a unique program of gene expression, lineage-specific transcription factors simultaneously exert inhibitory effects on alternate lineage gene programs by directly antagonizing the action of opposing transcription factors [140-142]. This means that a particular cell type will express a subset of transcription factors characteristic for the lineage, and these will positively regulate genes specifically expressed in the lineage. On another level these transcription factors specific for one lineage will repress genes of another lineage. This cross-antagonism of different lineage-specific transcription factors has been shown for PU.1 and GATA-1 (see figure 5).

Transcription factors in hematopoiesis

Different transcription factors have been implicated in the cell-fate decisions made at different time-points during hematopoiesis. Two of the transcription factors known to be essential for definitive hematopoiesis are c-Myb and AML1 (CBF-α). Both factors were first identified as oncoproteins, playing a role in a variety of different leukemias [143-145]. The core binding factors (CBF) are a small family of transcription factors comprising a DNA binding CBFα subunit and a non-DNA binding CBFβ subunit [146]. One gene encoding a CBFα subunit, RUNX1 (also known as AML1, CBFαA2, and PEBPA2A), and the gene encoding CBFβ are essential for hematopoiesis and are frequently mutated in human leukemias [145; 147]. The importance of AML1 in hematopoiesis has been shown by the phenotype of AML1 and CBFβ knockout mice. Both mice lack definitive hematopoiesis and die in utero, and embryonic stem cells from these null mice are unable to contribute to definitive hematopoiesis in chimeric mice. [148-152]. These findings demonstrate that AML1/CBFβ is essential for definitive hematopoiesis. However, its exact role in regulating the proliferation, self-renewal, and commitment of hematopoietic cells remains unknown. The CBF complex regulates the expression of various genes, including granulocyte-colony-stimulating factor (G-CSF) receptor, macrophage-colony-stimulating factor (M-CSF) receptor, myeloperoxidase, interleukin-3, and T-cell-receptor β gene [149-151].

c-Myb is highly expressed in immature hematopoietic cells and is down regulated during terminal differentiation of these cells [153; 154]. Knockout of the c-Myb gene results in embryonic lethality with a failure of hematopoiesis in fetal liver [155]. However, ectopic expression of c-Myb in the promyelocytic cell line 32Dcl3 permits G-CSF induction of primary granule protein myeloperoxidase although there is no further evidence of involvement in terminal differentiation [156]. This is in agreement with the idea that c-Myb stimulates cell proliferation and that cell cycle arrest is required for the terminal differentiation.

An important decision point in hematopoiesis for the common myeloid progenitor is the choice between the erythrocyte/megakaryocyte, the eosinophil/basophil, and the neutrophil/monocyte lineage. Two important transcription factors at this point are GATA-1 and PU.1 [141; 142; 157]. The GATA family of transcription factors contain an N-terminal activation domain and two conserved zinc fingers: the C-finger is capable of binding to DNA, whereas the N-finger stabilizes DNA binding. In addition to contacting DNA, the zinc-fingers also interact with another transcriptional co-factor, called Friend of GATA-1 (FOG-1) [158]. Within hematopoietic cells, expression of GATA-1 is limited to the erythroid, megakaryocyte, eosinophil, and basophil lineages and progenitors [159]. Targeted disruption of the mouse GATA-1 gene has provided strong evidence for its role as a key regulator of erythrocyte and megakaryocyte differentiation. Many erythroid-expressed genes contain GATA-motif sequences. GATA-1 is important in erythropoiesis and megakaryopoiesis and direct interaction with FOG-1 is required for terminal erythroid and megakaryocyte maturation. Ectopic expression of GATA-1 in myeloid cell lines leads to trans-differentiation into eosinophils (at intermediate GATA-1 levels) or de-differentiation into multiple progenitors (at high

GATA-1 levels) [160; 161]. This indicates that down-regulation of GATA-1 is a pre-requisite for the proper execution of the gene expression program.

PU.1 is a member of the *Ets* transcription factor family, possessing a glutamine-rich trans-activating domain at its N-terminus, the Ets domain and a C-terminal DNA binding domain [162]. *Ets* proteins bind to DNA as monomers and mediate protein-protein interactions that are only stable in the presence of DNA. DNA binding introduces conformational changes in PU.1 and exposes protein interaction domains. PU.1 is the most abundant Ets family member expressed in myeloid cells. Since almost all myeloid promoters have a functional PU.1 site close to the transcriptional start site, PU.1 is an important regulator of myeloid gene expression. Mice lacking PU.1 die one to three days before their expected birth [163]. The principal hematopoietic effects are absence of monocytes, neutrophils, eosinophils, and B and T lymphocytes.

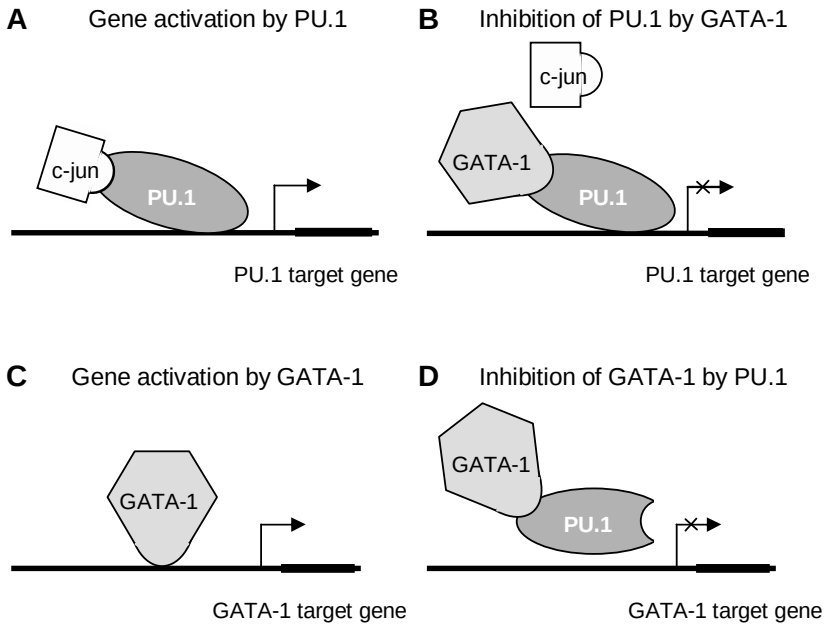

Figure 4. Antagonism between PU.1 and GATA-1. A Interaction between PU.1 and the co-factor c-Jun is required for expression of PU.1 target genes. **B** GATA-1 blocks the interaction between c-Jun and PU.1 by binding to PU.1. **C** Activation of GATA-1 target genes requires the binding of GATA-1 with DNA. **D** PU.1 binds to the DNA-binding domain of GATA-1, thereby blocking the activation of GATA-1 target genes.

There is also cross-antagonism between the lineage specific transcription factors GATA-1 and PU.1. Both transcription factors are expressed at low levels in multipotential precursor cells [164; 165]. Under direction of signals that are as yet undefined, GATA-1 or PU.1 is up regulated. Once GATA-1 is up regulated this leads to transactivation of its own promoter and stimulation of the erythropoietin receptor. However, when PU.1 is activated in a progenitor, this leads to stimulation of PU.1 through an auto-regulatory loop and triggers the expression of M-GSF, important for myeloid development. Recent studies have shown functionally important protein-protein interactions involving the lineage decision of the common myeloid progenitor [140-142]. GATA-1 inhibits PU.1 transactivation of PU.1 target genes by blocking the binding of the PU.1 co-activator c-Jun to the *Ets* domain of PU.1. The N-terminal region of PU.1 blocks GATA-1 function by binding to the GATA-1 C-finger and inhibiting GATA-1 binding to DNA (see figure 4).

C/EBP family

The CCAAT/enhancer binding protein (C/EBP) consists of six family members: α, β, γ, δ, ε, and ζ, which are part of a family of basic leucine zipper transcription factors. All C/EBP isoforms share substantial sequence identity (>90%) in the carboxy-terminal 55-65 amino acids residues, which contains the bZIP domain [166-168]. The leucine zipper consists of four or five repetitive leucine residues spaced every seven amino acids that result in an α helical amphipatic structure with hydrophobic and hydrophilic faces on each site of the helix. The leucine zipper is directly involved in homo- and heterodimerisation by a parallel coil-coil interaction and dimerisation is a pre-requisite for DNA-binding [167; 168]. Just amino terminal to the zipper is a basic region that is highly positively charged and directly interacts with DNA. C/EBP dimers bind to the consensus target site TTNNCCAAC. The promoters of many myeloid specific genes contain the C/EBP consensus-binding site, including granule proteins and cytokine promoters and are targets for transcriptional regulation by C/EBP family members. In contrast to the bZIP domain, the N-termini of the C/EBP proteins are quite divergent (<20% sequence identity) except for three short sub-regions that are conserved in most members [102]. These sub-regions represent the activation domains that interact with components of the basal transcription machinery and stimulate transcription [169-173].

The founding member of this family, C/EBPα was first identified to be highly expressed in liver and adipose tissue [174; 175]. C/EBPβ (NF-IL-6, LAP, IL6-DBP, NR-M) was originally described as a nuclear factor that binds the IL-1 responsive element in the IL-6 promoter [176; 177]. Constitutive expression of C/EBPδ is detected in intestines, adipose, and lung, however, high levels of expression are found in all tissues following LPS stimulation [178-180].

Coordinate expression of specific C/EBP isoforms is essential for normal hepatic synthetic activity and response to injury, C/EBPα is the predominant nuclear signal regulating terminal hepatocyte differentiation and function. Elimination of C/EBPα in mouse knockout models results in profound derangement of liver structure and function and death from hypoglycaemia within eight hours of

birth [181; 182]. Low level expression of phosphoenolpyruvate carboxykinase and perinatal lethality is also seen in a subset of C/EBPβ knockout mice, suggesting involvement of the C/EBPβ isoform in gluconeogenic pathways [183].

Mice deficient for both C/EBPβ and C/EBPδ die perinatally, similar to C/EBPα knockout mice [184]. C/EBPβ/δ double knockout mice did not accumulate lipid droplets in brown adipose tissue and had significantly reduced epididymal fat pads in surviving adults [184]. Despite these defects, C/EBPα and PPARγ (peroxisome proliferator-activated receptor) expression was normal, suggesting that C/EBPα and PPARγ are not sufficient for adipocyte differentiation in the absence of C/EBPβ and C/EBP δ [184].

C/EBPγ is ubiquitously expressed and the highest levels are found in non-differentiated, progenitor cells [185-187]. C/EBP ζ (also named CHOP) was first isolated as growth arrest and DNA-damage inducible gene 153 (*gadd153*) and is, like C/EBP γ, ubiquitously expressed [188-190]. C/EBPγ contains the basic and leucine zipper motifs that characterize the C/EBP family, but lacks the transcriptional activation domain present in the other family members [187]. C/EBPζ possesses a leucine zipper dimerisation domain and a DNA binding region, however, the presence of two prolines in the DNA binding region disrupts its helical structure and prevents heterodimer binding to C/EBP enhancer sequences. Therefore C/EBPζ and C/EBPγ function as inhibitors of C/EBP transcriptional activation [190].

C/EBP transcription factors demonstrate a unique temporal pattern of isoform expression in the myeloid lineage [191]. Within the hematopoietic system, C/EBPα expression is restricted to the neutrophil, monocyte, and eosinophil lineage. C/EBPα is highly expressed in proliferating myelomonocytic cells upon induction of differentiation and maintained during eosinophil and neutrophil differentiation. C/EBPβ levels are low in immature myeloid cells, increase after stimulation with IL-6, LPS, and IL-1, and are highest in macrophages and neutrophils [191; 192]. C/EBP,ε the most recently cloned member of the C/EBP family, is only expressed in myeloid lineages, with some low but detectable expression in the lymphoblastic cell line MOLT4 and the ovaries [193; 194]. C/EBP isoforms are not detected in either erythroid or lymphoid cells. The formation of tissue-specific combinations of C/EBP homodimers and heterodimers may allow this family of transcription factors to regulate different sets of genes in adipocytes, hepatocytes, and myeloid cells.

In the C/EBP α knockout mice, immature myeloid cells were found to be present in the peripheral blood in the absence of mature neutrophils or eosinophils [195]. Development of other hematopoietic lineages, including monocytes and macrophages was not affected. These findings demonstrate that disruption of C/EBPα produces a selective block in the development of mature granulocytes. Co expression of C/EBPα with other myeloid transcription factors synergistically activates myeloid-specific promoters.

The low basal DNA-binding activity of C/EBPβ is markedly increased by inflammatory stimuli, which induce phosphorylation and translocation to the nucleus, where it can activate multiple cytokine gene promoters [177; 196; 197]. Studies of mice with a targeted disruption of C/EBPβ gene

have demonstrated the importance of C/EBPβ for macrophage functions related to bacterial killing and tumor cytotoxicity. Nevertheless myeloid cell development was not adversely affected, consistent with a role of C/EBPβ in cytokine activation rather than lineage development [198; 199]. C/EBPβ deletion may be compensated by the presence of other C/EBP family members during early myelopoiesis. C/EBPβ is thought to play a role in the regulation of the eosinophil-specific MBP promoter. However in knockout mice there is no indication that it is involved in the regulation of eosinophil differentiation [200]. C/EBPδ deficient mice show no phenotypic abnormalities in hematopoietic lineages.

In contrast to C/EBPα acting early in myeloid differentiation, C/EBPε is involved more in terminal differentiation [166; 193; 201]. C/EBPε nullizygous offspring are normal at birth and fertile. However, they fail to produce normal neutrophils or eosinophils [202]. Median survival of C/EBPε-deficient mice is 50 to 75 days with the usual cause of death due to opportunistic infection with *Pseudomonas aeruginosa* with concomitant tissue destruction [202]. Additionally, peripheral blood white cell differentials in young mice (two weeks of age or less) show only a slight increase in less mature myeloid cells in knockout animals compared with wild-type mice [202]. Conversely, older mice (three to four months of age) display a pronounced increase in immature myeloid forms in both their peripheral blood and bone marrow [202]. Although C/EBPε deficiency blocks the maturation of granulocytic lineages, there is no effect on both erythropoiesis and lymphopoiesis.

Transcription factors in eosinophil differentiation

Work by Graf and Nerlov has contributed considerably to understanding the role of transcription factors in eosinophilopoiesis. Using a model system they focused on the differentiation of avian multipotent hematopoietic progenitors (aMEPs) and have demonstrated that a complete down-regulation of GATA-1 causes MEPs to differentiate into the neutrophil/monocyte lineage. In contrast, intermediate levels correlate with the formation of eosinophils and high levels with that of thromboblasts and erythroblasts [160]. The stabilization of GATA-1 expression at an intermediate level is likely to be instrumental in the decision of a multipotent progenitor to choose the eosinophil over the neutrophil/monocyte lineage.

They also showed that both the α and β isoforms of C/EBP are able to induce eosinophil lineage commitment of aMEPs. C/EBP can regulate genes involved in eosinophil lineage commitment independently of its transactivation function. They showed that all C/EBP proteins capable of DNA-binding promote eosinophil lineage commitment, whereas only those with an intact transactivation domain supported terminal differentiation [203]. The results of the C/EBPα and C/EBPε knockout mice and the C/EBP induced eosinophil differentiation provide a strong indication for an important role of C/EBP in eosinophil lineage commitment. Taken together, the forced expression of C/EBPs in aMEPs and of GATA-1 in myeloid cells both lead to eosinophil differentiation 160; 203. Therefore, C/EBP and an intermediate level of GATA-1 are likely to be sufficient to specify the eosinophil lineage.

FOG has been shown to play an essential role in the maturation of erythroid cells and in the early development of the thrombocytic lineage [158]. FOG is highly expressed in multipotent hematopoietic progenitors, but not in their derived myeloid and eosinophil cells [140; 158; 200]. Recent data showed that ectopic expression of FOG in an eosinophilic cell line led to down regulation of eosinophil specific markers and up regulation of MEP markers, indicating that FOG is a repressor of the eosinophil lineage. C/EBP-mediated down-regulation of FOG is a critical step in eosinophil lineage commitment, indicating that loss of FOG expression is a pre-requisite for eosinophil gene expression. Maintenance of a multipotent state in hematopoiesis is achieved through cooperation between FOG and GATA-1. FOG repressed the expression of an avian eosinophilic marker, EOS47, in a GATA-1 dependent manner. Taken together, C/EBP repress transcription of the FOG gene, whereas FOG acts as a co-repressor with GATA-1 to inhibit transcription of C/EBP target genes (see figure 5).

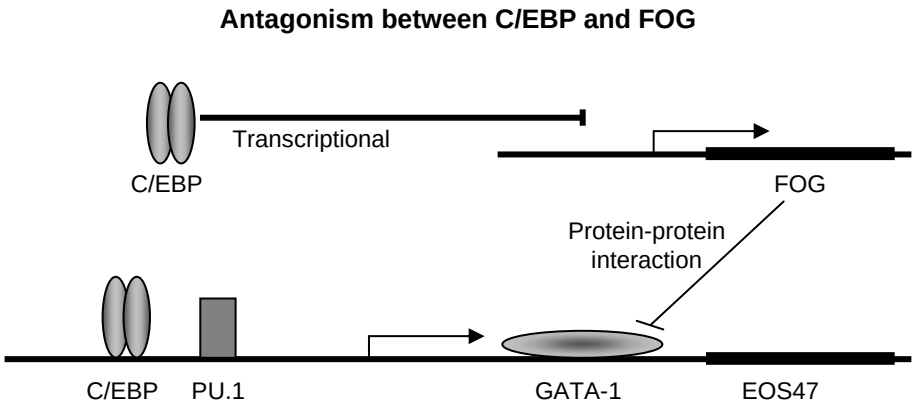

Figure 5. Antagonism between C/EBP β and FOG. C/EBPβ represses FOG expression at the transcriptional level. It is not known whether this occurs by a direct mechanism or through C/EBPβ target genes. FOG acts as a transcriptional co-repressor of gene activity of C/EBPβ target genes through protein-protein interaction with GATA-1.

Scope of this thesis

Hematopoiesis is the coordinated production of mature blood cells from a common stem cell via different stages of commitment and differentiation. The development of the different branches is regulated by transcription factors, cytokines and growth factors. Factors regulating cell-specific gene expression are also important in the control of cell differentiation. The development of eosinophils is regulated by transcription factors, cytokines, and growth factors.

Research questions

How is the expression of eosinophil specific proteins regulated?

In chapter 2 the regulation of the eosinophil specific granule protein EDN is analyzed. Chapter 3 and 4 describe the gene regulation of both chains of the IL-5 receptor of which the IL-5Rα is eosinophil-specific and the shared β-chain is expressed in all myeloid cell types.

What is the role of C/EBP proteins on granulocyte differentiation?

The effect of a dominant-negative variant of C/EBP proteins in the differentiation $CD34^+$ cells derived from human umbilical cord blood towards eosinophils is described (Chapter 5). In chapter 6 the expression of C/EBPε on G-CSF induced granulocytic differentiation is investigated.

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Chapter 2

C/EBP regulates the promoter of the eosinophil derived neurotoxin/RNS2 gene in human eosinophilic cells

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Journal of Leukocyte Biology 1999 66:683

Abstract

The eosinophil-derived neurotoxin (EDN), a member of the mammalian ribonuclease family, is found in the large specific granules of human eosinophilic leukocytes. We have investigated the role of the C/EBP transcription factor family in the regulation of EDN promoter activity. Here we show that the C/EBP family is involved in intrinsic regulation of EDN promoter activity. We have identified a C/EBP binding site located at –124 in the proximal promoter of the EDN gene. Mutation of this C/EBP site results in a decrease of promoter activity in HL-60-eos cells as well as in eosinophils differentiated in vitro from $CD34^+$ cells. Different C/EBP proteins are able to bind to the C/EBP site as shown by gel shift assay. Our results indicate the importance of the C/EBP family in the regulation of the EDN gene in eosinophils.

Introduction

Eosinophils play an important role in the protection against parasites, and are also involved in the pathogenesis of allergic diseases, such as allergic asthma and atopic dermatitis [1, 2]. Parasite killing and tissue damage found in allergic lesions are both linked to production of toxic oxygen metabolites and to the release of eosinophil granule proteins by degranulation or exocytosis [2-4]. Eosinophil-derived cytotoxic proteins include major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil derived neurotoxin (EDN) [1,5].

EDN is localized in the granule matrix of human eosinophils and possesses neurotoxic, helmintoxic, and ribonucleolytic activities [6]. EDN is synthesized as a pre-protein of 161 amino acids including a 27 amino acid signal peptide, which is processed to form the mature protein [7-10]. The EDN gene consists of two exons separated by a single intron. The second exon contains the complete coding sequence and 3'-untranslated sequences. These structural features are shared with other genes of the RNAse gene superfamily, such as ECP. EDN mRNA can be detected in the RNA from hypodense eosinophils [9] and during the pro-myelocytic stage of eosinophil development as judged by in vitro differentiation of cord blood mononuclear cells [11]. Similarly, EDN mRNA expression can be detected in the pro-myelocytic HL-60 cell line when it is induced to differentiate with interleukin-5 (IL-5) or butyric acid (BA) [9,12,13].

Previously it was shown that optimal expression of EDN in HL-60 cells is dependent on the interaction of the proximal promoter sequences and the intron [12]. The promoter and the first intron contain multiple consensus transcription factor-binding sites. Recently, it was shown that the nuclear factor of activated T cells (NF-AT) site plays an important role in the activity of the intronic enhancer in undifferentiated HL-60 cells [14], while the intronic PU.1 site is involved in the regulation of the gene in the eosinophil-differentiated HL-60-eos cells [13]. Over the last years several myeloid-specific genes and their regulatory sequences were characterized. A number of transcription

factor families have been shown to be important for the transcription of myeloid specific genes, including the C/EBP family, the Ets family and cMyb [15-17].Proteins of the rapidly expanding C/EBP family belong to the basic domain/leucine zipper class of transcription factors, which bind to DNA as homo- and heterodimers with family members or unrelated transcription factors (reviewed in [18]). C/EBPα was initially identified in liver and adipose tissue, where it was found to be important for terminal differentiation [19, 20]. More recently, it was shown C/EBPα was also expressed in early myeloid cells and was involved in the regulation of a number of granulocyte-specific genes [16,21,22].Mice deficient in C/EBPα display an early block in the maturation of granulocytes [23]. Both C/EBPβ and C/EBPδ play a role in the mediation of the inflammatory response [24, 25]. C/EBPγ is ubiquitously expressed with highest levels found in non-differentiated progenitor cells. C/EBPγ lacks transcriptional activation elements, suggesting a dominant negative regulation of C/EBP transactivation in undifferentiated cells [26, 27]. C/EBPε is essential for terminal differentiation and functional maturation of committed granulocyte progenitor cells [28, 29].

In the present study, we have investigated the role of C/EBP in the regulation of the EDN gene during eosinophilic differentiation. Our results demonstrate that C/EBP is involved in the regulation of EDN transcription by binding to a site in the upstream promoter. Mutation of this C/EBP site strongly decreases the activity of the EDN promoter in HL-60-eos as well as in eosinophils differentiated in vitro from $CD34^+$ progenitors.

Materials and Methods

Cell culture, reagents, and antibodies

HL-60 cells, a promyelocytic leukemia cell line, were cultured in RPMI 1640 (Life Technologies, Breda, The Netherlands.) supplemented with 8% fetal calf serum, (FCS, Hyclone), 100 U/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies) and 50µM β-mercaptoethanol. HL-60 7.7 cells were generated by culturing HL-60 cells for two months at pH 7.7 in RPMI containing 25 mmol/L N-[2-Hydroxyethyl]piperazine-N'-3-propane-sulfonic acid (EPPS; Sigma, St Louis, MO). To further differentiate HL-60 7.7 cells, butyric acid (0.5mmol/L; Sigma) was added 4 to 6 days (HL-60-eos cells).

$CD34^+$ cells were isolated from cord blood and differentiated as described by Caldenhoven et al. [30]. In short, mononuclear cells (MNC) were obtained by centrifugation of the blood on a Ficoll-Hypaque gradient (density 1.077 g/ml). MNC cells were incubated with $CD34^+$ antibody and magnetic beads, prior to positive selection on a column which was placed in a magnetic field. Cells were cultured in Iscove's modified Dulbecco medium (IMDM; Life Technologies) supplemented with 10% FCS, SCF (100 ng/ml), FLT-3 ligand (100 ng/ml), IL-3 (0.1nmol/l), GM-CSF (0.1nmol/l), and IL-5 (0.1nmol/l). After three days, cells were cultured in IMDM supplemented with 10%

FCS, IL-3 and IL-5. From day 21, IL-3 was also omitted from the media. Viability remained >95% throughout the 28 day experiment.

COS-1 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 8% heat inactivated fetal calf serum (FCS; Life Technologies), 100 U/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies).

The following antibodies were used in this study: anti-C/EBPα (rabbit polyclonal IgG 14aa, sc-061X; Santa Cruz), anti-C/EBPβ (rabbit polyclonal IgG C-19, sc-150X; Santa Cruz), anti-C/EBPδ (rabbit polyclonal IgG M-17, sc-636X; Santa Cruz) anti-C/EBPε (rabbit polyclonal IgG C-22, sc-158X; Santa Cruz) and C/EBPβ Δ198 (rabbit polyclonal IgG Δ198, sc-746X; Santa Cruz).

Synthetic oligonucleotides and plasmid construction

The EDN promoter/intron (-300 to +298, GENBANK X16546) was cloned by PCR using human genomic DNA [13]. The amplified fragment was then sequenced and cloned into the promoterless CAT reporter plasmid pBLCAT3. Deletion constructs were generated by PCR and cloned into pBLCAT3.Transcription factor binding sites were identified using the TFSEARCH program (http: //pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html).

The following oligonucleotides were used in this study: for site directed mutagenesis Δ-144: TCCTTTTTATTCCATGCCATGCCTTGGCTGAG; Δ-124: AGTCCACACGTGATATCATCC TTTT T; Δ+163: CTGCAAAGACTGACGCGTCTCCTAAGC; for bandshift analysis (only the upper strands are shown): -124: AGCTTATGATTGCACAAGTG; Δ-124: AGCTTATGATAT CACGTGTG; somatostatin CRE [31]. Site-directed mutagenesis was performed as described previously [32]. Human C/EBPα (a kind gift from Dr. G.W.M. Swart), human C/EBPβ (a kind gift from Prof. S. Akira), human C/EBPγ (cloned by PCR from HL-60 cDNA), rat C/EBPδ (a kind gift from Prof. S.L. McKnight), human C/EBPε (cloned by PCR from HL-60 cDNA), and DN/C/EBP (a kind gift from Dr C. Nerlov) were cloned into the expression vector pLNCX (Clontech).

Transient transfection and CAT assay

For transfection experiments, COS-1 cells were cultured in six-well dishes (Nunc, Naperville, IL), and 3 hrs later the cells were transfected with 6-8 μg supercoiled DNA as described previously [33]. Following 16-20 hrs exposure to the calcium-phosphate precipitate, medium was refreshed. Cells were harvested for CAT assays 24 hrs later. Transfection of HL-60-eos cells and in vitro differentiated eosinophils was performed by electroporation at 300V and 960μF (10^7 cells, 20 μg supercoiled plasmid DNA). Two days after transfection cells were harvested for CAT assays. CAT assays were performed as described previously [34]. CMV-LacZ co-transfection and β-galactosidase assays were used to correct for variations in transfection efficiency.

Gel Retardation Assay

Nuclear extracts were prepared from the HL-60-eos line following a previously described procedure [35]. Oligonucleotides were labeled by filling in the cohesive ends with [α^{32}P] dCTP using Klenow fragment of DNA polymerase I (Life Technologies, Inc.). Gel retardation assays were carried out according to published procedures with slight modifications [36]. Briefly, nuclear extracts were incubated in a total volume of 20 µl, containing 10mM HEPES-KOH pH 7.9, 50mM KCl, 5mM $MgCl_2$, 1mM EDTA, 10% (v/v) glycerol, 5mM DDT, 20 µg BSA, 2 µg poly dI-dC (Pharmacia Biotech. Inc.) and 1 ng of a [α-^{32}P]-labeled EDN –124 oligonucleotide for 20 minutes at room temperature. In competition experiments, indicated molar excess of unlabeled oligonucleotide was added simultaneously with the [α-^{32}P]-labeled oligonucleotide. For supershift analysis, nuclear extracts were pre-incubated with antibodies against C/EBPs for 30 minutes on ice before the addition of [α-^{32}P]-labeled oligonucleotide. Protein-DNA complexes were then separated on 5% non-denaturing polyacrylamide gels and visualized by autoradiography.

Results

It was previously shown that EDN transcription is dependent on sequences located upstream of the first exon as well as in the first intron [12, 13]. Several sites in the intron have been reported to be important for gene regulation. So far there are no sites in the 5' upstream region identified to play a role in the regulation of EDN. To investigate the role of C/EBP proteins in the regulation of the EDN gene we co-transfected HL-60-eos cells with an EDN promoter/intron CAT reporter construct and C/EBPγ or a dominant negative form of C/EBP. C/EBPγ lacks transcriptional activation elements, and therefore acts as a dominant negative regulator of C/EBP transactivation. The dominant negative C/EBP (DN/C/EBP) can dimerise with all C/EBP family members, but is mutated in the DNA binding domain and therefore unable to bind to DNA. Figure 1A shows that co-transfection of C/EBPγ reduces EDN promoter activity with 70%, while DN/C/EBP reduces promoter activity with 90%. These results strongly suggest a role for C/EBP proteins in the regulation of EDN transcription in HL-60-eos cells.

To examine if the EDN promoter/intron can also be activated by C/EBP proteins in other cell systems, we co-transfected different C/EBP family members with the EDN promoter/intron in COS cells. As shown in figure 1B, both C/EBPα and -δ greatly increase CAT activity, whereas C/EBPβ and ε increase the activity of the EDN promoter to a lesser extent. These results show that the activity of the EDN promoter can be enhanced by C/EBP family members and that C/EBPα and -δ are the most potent inducers of EDN transcription.

Computer-assisted analysis of the EDN promoter indicates the presence of three potential C/EBP binding sites centered around -144, -124, and +163. To determine which of the C/EBP sites are involved in the regulation of the EDN promoter, we constructed a number of deletion constructs,

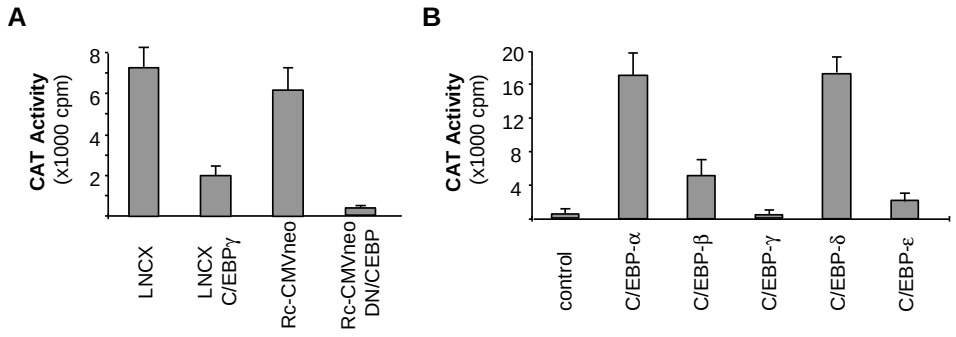

Figure 1. C/EBP activates the EDN promoter. A. HL60-eos cells were transfected by electroporation with the EDN promoter CAT construct (5 μg) in combination with 15 μg of either LNCX-CEBPγ, a C/EBP member that lacks the transactivation domain, the empty LNCX vector, a synthetic dominant negative (DN) C/EBP or the empty Rc-CMVneo expression vector. Transfection of either C/EBPγ or DN-C/EBP clearly reduces EDN promoter activity in HL60-eos cells. The activity of the EDN promoter CAT construct is roughly 10-fold higher that the activity of the promoterless CAT vector pBLCAT3 (data not shown). **B.** The EDN promoter (2μg) was co-transfected with different C/EBP family members (4μg) in COS-1 fibroblasts by the calcium phosphate precipitation method. Bars indicate the mean value of at least three independent experiments, standard deviation is indicated by error bars. CMV-LacZ co-transfection and β-galactosidase assays were used to correct for variation in transfection efficiency. The EDN promoter can be activated by C/EBPα, β, δ, and ε.

which are schematically shown in figure 2A. The responsiveness of these constructs to C/EBP co-transfection was then tested in COS cells. Figure 2B shows there was a slight reduction of C/EBP induced promoter activity when C/EBP -144 was deleted (-128 - +173). However, deletion of the regions containing C/EBP –124 (-95 - +173) or C/EBP +163 (-167 - +123) leads to a stronger decrease of promoter/intron activity in C/EBP transfected cells. These results show that the putative C/EBP binding sites C/EBP -124 and C/EBP +163 might be involved in the regulation of the EDN promoter.

To investigate if the putative C/EBP binding sites are also involved in eosinophilic cells containing endogenous C/EBP proteins, we transfected the deletion constructs in HL-60-eos cells (fig. 3A). Deletion of the region –167 to –128, containing the putative C/EBP –144 binding site, slightly reduces promoter/intron activity, whereas deletion of the regions -128 to –95 or +123 to +173, which contains either the C/EBP –124 or the C/EBP +163 binding site show a significant decrease in promoter/intron activity. However, the deletion construct –95 - +173, containing only C/EBP +163 shows a low activity. These results suggest that C/EBP family members play an important role in EDN promoter regulation, and that C/EBP –124 binding site seems to be more important than the C/EBP +163 site.

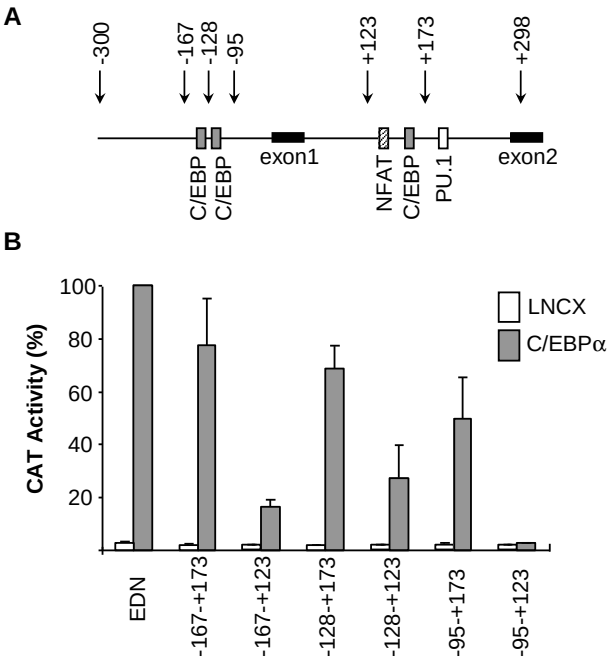

Figure 2. Mapping of the C/EBP responsive sequences in the EDN promoter. A. Schematic representation of the EDN promoter and intron. Putative transcription factor binding sites are indicated as boxes. The arrows indicate the 5' ends and the 3' ends of the deletion constructs used in panel B. **B.** COS-1 cells were transfected with deletion constructs of the EDN promoter and the first intron cloned into the promoterless pBLCAT3 (2μg) together with LNCX (control) or LNCX-C/EBPα (4μg). Two days after transfection cells were harvested and CAT activity was determined. Bars indicate the mean value of at least three independent experiments, standard deviation is indicated by error bars. CMV-LacZ co-transfection and β-galactosidase assays were used to correct for differences in transfection efficiency. Deletion of the C/EBP -124 or C/EBP +163 sites results in a decrease in C/EBP-induced promoter activity.

To further study the importance of the C/EBP sites for the promoter /intronic enhancer activity, we prepared different point mutations in the background of the complete promoter/intron sequence. We mutated the first C/EBP site (C/EBP Δ-144), the second (C/EBP Δ-124) or the third (C/EBP Δ+163) or all three of them (C/EBP 3Δ). The activity of the different mutants was then tested in HL-60-eos cells (fig.3B). Mutation of C/EBP -144 or C/EBP +163 had no effect on promoter/intron activity, while mutation of C/EBP -124 significantly decreases the activity of the EDN promoter/intron. Activation of the EDN promoter is not further reduced after mutation of all three putative C/EBP binding sites in comparison with the single mutation of C/EBP -124. These results indicate that activation of the EDN promoter/intron by C/EBP proteins is regulated by C/EBP -124. We also transfected the different mutants of the EDN promoter/intron in eosinophils

differentiated in vitro from cord blood $CD34^+$ cells. As in HL-60-eos cells, there was no effect on promoter activity after mutation of -144 or +163. However, there was a significant decrease in activity after mutation of the C/EBP -124 site (fig. 3C). Mutation of all three C/EBP sites resulted in a reduction of promoter activity comparable to the single mutation of the C/EBP –124 site. These results indicate the importance of the C/EBP -124 site in eosinophils.

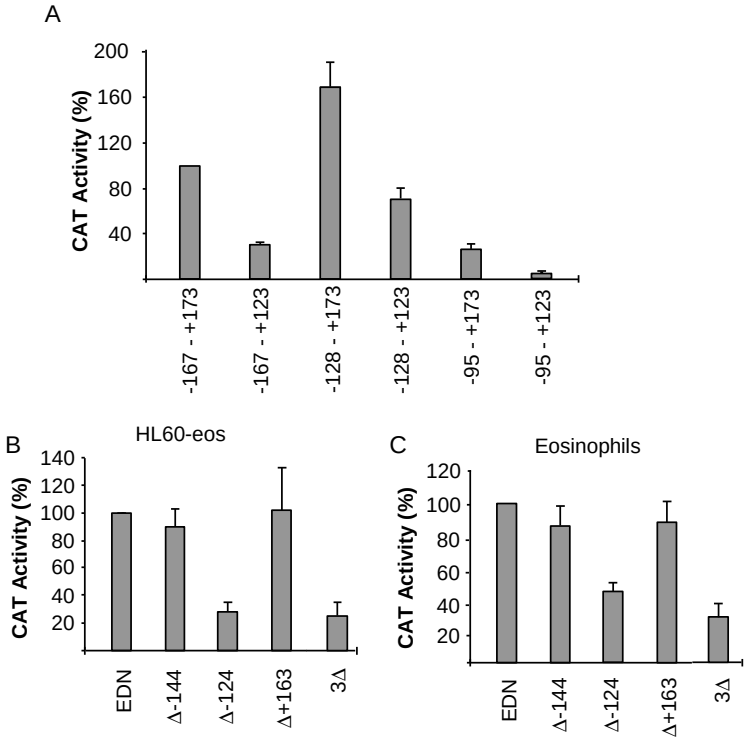

Figure 3. The C/EBP binding site at –124 is essential for EDN promoter activity in eosinophilic cells. A. Deletion constructs of the EDN promoter and the first intron cloned into the promoterless pBLCAT3 (see legend 2A) (20μg) were transfected in HL-60 –eos cell s by electroporation. Two days post-transfection, CAT activity was determined. Deletion of the C/EBP -124 or C/EBP +163 sites results in a decrease in promoter activity. **B.** EDN-CAT constructs (20μg) containing the full promoter and intron with different point-mutations in the C/EBP sites were transfected in HL-60-eos cells. This figure shows that the C/EBP -124 binding site is crucial for promoter activity in HL60-eos cells. **C.** EDN-CAT constructs (20 μg) as described in B were transfected in eosinophils differentiated in vitro for 17 days as described under Materials and methods. The activity of the EDN promoter CAT construct is roughly 10-fold higher that the activity of the promoterless CAT vector pBLCAT3 (data not shown). As in HL60-eos cells, the C/EBP -124 binding site is also crucial for promoter activity in eosinophils.
Bars indicate the mean value of at least three independent experiments, standard deviation is indicated by error bars. CMV-LacZ co-transfection and β-galactosidase assays were used to correct for transfection efficiency.

Band shift analysis was used to investigate whether C/EBP proteins can interact with the C/EBP -124 site. An oligonucleotide probe, containing the C/EBP -124 site, was end labeled and incubated with 10 μg nuclear extract of HL-60-eos cells. As shown in figure 4 the C/EBP -124 site was able to bind several protein complexes (lane 1). Binding of the observed complexes was specific because binding could be competed with a 10-100 molar excess of cold self-oligo (lanes 2 and 3). In contrast the binding could not be competed by the unrelated cAmp response element (CRE) site (lane 4). To further identify the proteins binding to C/EBP -124, extracts were pre-incubated with specific antibodies against Ets2 or different C/EBP family members, including α, -β, -ε. Antibody β Δ198 is able to bind all C/EBP family members. Figure 4 shows that all C/EBP antibodies induce a shift of the protein complex (lanes 6-9), whereas the unrelated Ets2 antibody is not able to induce a super shift (lane 5). These results indicate that C/EBPα, β, and ε proteins are able to bind to C/EBP –124 of the EDN promoter in HL-60-eos cells

Taken together, these results strongly suggest that multiple members of the C/EBP family are capable of interacting with the C/EBP site of the EDN promoter

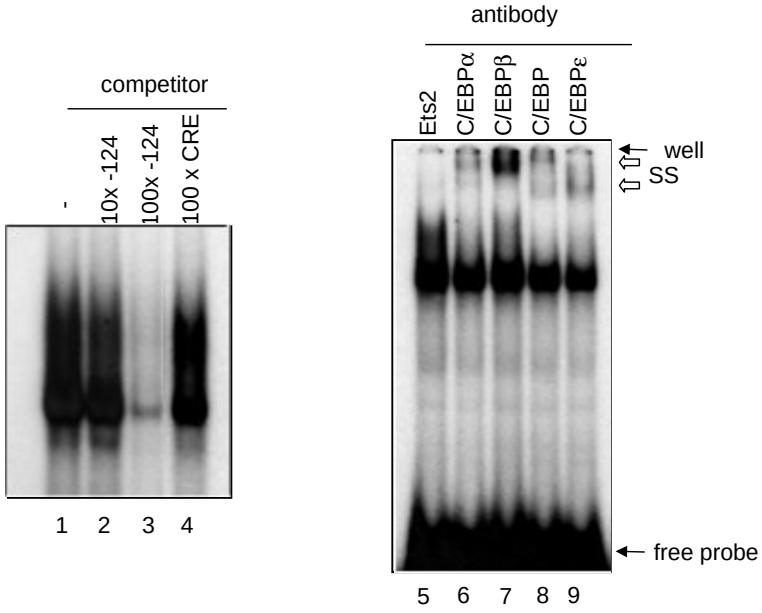

Figure 4. Binding of different C/EBP proteins to the EDN promoter. Nuclear extracts were prepared from HL-60-eos cells. These extracts were then tested for C/EBP binding activity in a band shift assay using the –124 binding site as a probe. Lane 1 shows the binding of a complex binding to C/EBP -124, which can be competed with cold self-oligo (lanes 2 and 3), but not with the unrelated CRE site (lane 4). In lanes 1-4, only the shifted complex is shown. The DNA-protein complex can be supershifted (SS, open arrows) with antibodies against C/EBP α, β, and ε and an antibody recognizing all C/EBP family members (lanes 6 to 10), but not with Ets antibodies (lane 5). The free DNA probe and the wells of the gel are indicated by closed arrows.

Discussion

In this paper we show that C/EBP transcription factors are involved in the regulation of the EDN promoter. C/EBP proteins were previously implicated in the regulation of the myeloid colony stimulating factor receptors, like M-CSF[37], G-CSF [38] and the common β chain of the IL-3/IL-5/ GM-CSF receptors [53], as well as in the regulation of some myeloid specific granule proteins, like MPO, neutrophilic elastase and lysozyme M [39,40]. Importantly, this is the first report describing a role for C/EBP proteins in human eosinophil-specific transcription, although the involvement of C/EBPs in chicken eosinophil-specific gene regulation was recently reported [41].

Members of the C/EBP family have been shown to have a changing temporal expression pattern during myeloid differentiation [42, 43]. C/EBPα is expressed at high levels in early progenitors, C/ EBPβ and -δ become more prominent at later stages, while C/EBPε is only expressed during granulopoiesis [28,29]. Alternative splicing and the ability of all members of the C/EBP family to form hetero- and homodimers as well as complexes with unrelated transcription factors lead to a variety of the possible DNA binding complexes [37,44-49]. It is likely that these complexes have specific affinities for particular binding sites and that this mechanism plays an important role in the specific regulation of certain genes by the C/EBP family. Selective expression of the different family members may also play a role in determining which C/EBP protein is important at a particular stage of differentiation. Different members of the C/EBP family can stimulate activity of the EDN promoter/intron in an overexpression system (Fig. 1B). However, our bandshift results show that in HL60-eos cells, which express endogenous EDN and C/EBP, C/EBPα, β, and ε interact with the EDN promoter. Since C/EBPα and ε have been shown to be important for granulocyte differentiation and are highly expressed in mature granulocytes, it is likely that these two are the most important regulators of EDN transcription in eosinophils. However, a more detailed analysis is required to determine the precise contribution of each C/EBP family member to EDN transcription in eosinophils.

We have shown that the C/EBP -124 site in the promoter of the EDN gene can bind C/EBP proteins (fig 4). This is essential for optimal activity, since mutation of this site results in reduced promoter activity (fig 3B). However, there is significant residual promoter/intron activity after mutation of the –124 C/EBP site. This is likely due to activity of the PU.1 site, which we have previously shown to be involved in the regulation of the EDN gene [13]. In the last few years it has become clear that the C/EBP family and PU.1, a hematopoietic-specific member of the Ets family, are key transcription factors in the regulation of myeloid-specific genes, including myeloid CSF receptors and myeloid granule proteins [37-40]. Moreover, PU.1 and C/EBP proteins have also been shown to co-operate during eosinophilic differentiation in mice and chicken [17,37,50-52]. In a previous study we have shown that the EDN gene is regulated by PU.1 [13]. In this study we report that C/EBP proteins also play a role in EDN expression, by binding to an upstream enhancer. This is the first evidence of the role of C/EBP and PU.I in the regulation of an eosinophil-specific

gene. Interestingly, the C/EBP site is located in the upstream promoter and the PU.1 site in the first intron, separated by 300 bp. Previous studies have shown the co-operation in gene regulation between PU.1 and C/EBP proteins whose binding sites were located close to each other [37, 38]. However co-operation can also occur between C/EBP and PU.I sites separated by as much as 2kb, as we have recently shown for the promoter of the human IL-3R/IL-5R/GM-CSFR β chain [53].

In COS fibroblasts both the C/EBP -124 and the C/EBP +163 sites seem to be responsive to C/EBPα co-transfection. However, in the hematopoietic cells, HL-60-eos and in vitro differentiated eosinophils, only the C/EBP -124 site is involved in EDN promoter activity. This difference might well be due to the overexpression of C/EBPα in the COS cells which might render the +163 site responsive, whereas the activation of the EDN promoter in HL-60-eos cells and eosinophils is regulated by moderate endogenous levels of C/EBP.

Taken together we have shown the involvement of C/EBP proteins in the regulation of the eosinophil-specific granule protein EDN. This is the first example of eosinophil-specific gene regulation by C/EBP and PU.I. However, other eosinophil-specific genes are also expected to be regulated by these proteins, since both PU.1 and C/EBP (α and ε) are necessary for the in vivo differentiation of eosinophils.

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Chapter 3

An AP-1 site in the promoter of the IL-5Rα gene is necessary for promoter activity in eosinophilic HL-60 cells

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FEBS Letters 1998 434: 251

Abstract

Interleukin-5 (IL-5) plays a crucial role in the proliferation, differentiation and activation of eosinophils. The IL-5 receptor is composed of an IL-5-specific α subunit, which is expressed by eosinophils and basophils, and a βc-subunit shared with the receptors for IL-3 and GM-CSF. We identified an AP-1 element which is important for IL-5Rα promoter activity. The AP-1 site and the previously identified EOS1 site cooperate, since single mutation of either of the sites decreased promoter activity. We show that the AP-1 site of the IL-5Rα promoter binds multiple proteins, including cJun, CREB, and CREM.

Introduction

Cytokines such as interleukin-3 (IL-3) and GM-CSF are involved in the early differentiation of granulocytes, whereas the terminal differentiation of eosinophils selectively depends on IL-5 [1; 2]. Since a functional IL-5 receptor is only expressed on basophils and eosinophils, IL-5 controls the proliferation, differentiation, and functioning of eosinophils [3; 4]. The IL-5 receptor (IL-5R) consist of two distinct membrane proteins, the α and β chain. The α-chain (IL-5Rα) is unique for the IL-5R, is restricted to eosinophils and basophils and binds IL-5 with low affinity [5-7]. Expression of the IL-5Rα gene may be critical to the entry of multipotential myeloid progenitors into a particular hematopoietic developmental program. The β chain is shared with the receptors for IL-3 and GM-CSF and is widely expressed in various hematopoietic lineages [8]. The β-chain does not bind IL-5 by itself, but is required for high affinity binding [5; 9; 10].

The gene encoding the human IL-5Rα chain is located on chromosome 3. Recently the 5' upstream region of this gene, containing multiple consensus sites for known hematopoietic transcription factors, was isolated, which appears to be highly active in the eosinophilic lineage [11]. Sun *et al*. [12] identified a unique enhancer-like cis element (EOS1) that is necessary and sufficient for promoter activity and binds an unknown transcription factor. Besides this EOS1 site the promoter contains several consensus sites for known transcription factors. In this study we show the involvement of an AP-1 site in the regulation of the IL-5Rα gene. The AP-1 family is a set of sequence specific transcription factors, including the Jun, Fos, and activating transcription factor (ATF, including CREB and CREM) families, which form a variety of hetero- and homodimers [13; 14]. Interestingly, AP-1 family members are known to be involved in the regulation of myeloid differentiation [15-17].

In this study we demonstrate that, besides the EOS1 site, the AP-1 element of the IL-5Rα promoter located between -440 and -432 is also involved in the regulation of the transcription. Therefore, the cooperative regulation of the unique EOS1 site and the AP-1 site is of great interest.

Materials and Methods

Cell culture, reagents, and antibodies

Promyelocytic leukemia-HL-60 cells were cultured in RPMI 1640 (Life Technologies, Breda, The Netherlands.) supplemented with 8% fetal calf serum, (FCS, Hyclone) 100 U/ml penicillin (Life Technologies, Inc.), 100 μg/ml streptomycin (Life Technologies, Inc.) and 50μM β-mercaptoethanol. HL-60 7.7 cells were generated by culturing HL-60 cells for two months at pH 7.7 in RPMI containing 25 mmol/L N-[2-Hydroxyethyl]piperazine-N'-3-propane-sulfonic acid (EPPS; Sigma, St Louis, MO). To further differentiate HL-60 7.7 cells butyric acid (0.5mmol/L; Sigma) was added up to 6 days. Polyclonal rabbit antibody against cJun was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against CREB and CREM were described previously [18].

Synthetic oligonucleotides and plasmid construction

The following oligonucleotides were used in this study (only the upper strands are shown): the AP-1 (5'-AGCTTAAATCATGTGTCAGTGTTGA-3'), AP-1 mutant (5'-AGCTTAAATCA TGGGCCCGTGTTGA-3'), Col-TRE (5'-GATCTATCTGAGTCAGCAG-3'), c-jun TRE (5'-GATCTATCTGACATCAGCAG -3'), and SP1 (5'-AGCTTGGGGCGGGGCT-3'). A human genomic DNA library (λ Fix II, Stratagene) was screened using a random primed IL-5Rα probe (provided by Dr. B. Allet, Glaxo Institute). Secondary and tertiary screening was performed using an oligo corresponding to most of the first exon (6). Isolated DNA fragments were cloned into the KpnI-NotI sites of the pBKS II phagemid (Stratagene). Fragments of the IL-5Rα upstream sequence were generated using the polymerase chain reaction with primers containing Hind III and Pst I sites for sense and antisense sequences, respectively, and ligated into the pCAT basic vector (Promega). Site-directed mutagenesis was performed as described by Kunkel [19].

Electro Mobility Shift Assay

Nuclear extracts were prepared from the different HL-60 sublines following a previously described procedure [20]. Oligonucleotides were labeled by filling in the cohesive with [α^{32}P] dCTP using Klenow fragment of DNA polymerase I (Life Technologies, Inc.). Gel retardation assays were carried out according to published procedures with slight modifications [21]. Briefly, nuclear extracts were incubated in a total volume of 20 μl , containing 10mM HEPES-KOH pH 7.9, 50mM KCl, 5mM $MgCl_2$, 1mM EDTA, 10% (v/v) glycerol, 5mM DDT, 20 μg Bovine serum albumin (BSA), 2 μg poly dI-dC (Pharmacia Biotech. Inc.) and 1 ng of a [α-^{32}P]-labeled AP-1 oligonucleotide for 20 minutes at room temperature. In competition experiments, indicated molar excess of unlabeled

oligonucleotide was added simultaneously with the [α-^{32}P]-labeled oligonucleotide. Antibodies were incubated 30 minutes on ice prior to the addition of [α-^{32}P]-labeled oligonucleotide. Protein-DNA complexes were then separated on 5% non-denaturing polyacrylamide gels and visualized by autoradiography.

Transient transfection and CAT assay

HL-60 cells were transfected by electroporation (280 V, 960 μF). Transfected cells were harvest for CAT assay after 48 hour. Cells were lysed by repeated freeze thawing in 250mM Tris pH 7.4, 25mM EDTA Cellular extract was incubated in a total volume of 200 μl 200mM Tris pH 7.4, 4% glycerol, 0.5mg/ml Butyryl CoA and 0.05μCi [^{14}C]-Chloroamphenicol for two hours at 37°C. Reaction products where then extracted using 400 μl xylene/pristane (1:2), and the percentage of acetylated products was then determined using liquid scintillation counting. A CMV-lacZ construct was co-transfected to correct for differences in transfection efficiency.

RNA isolation and Northern blotting

For Northern blotting, RNA (20μg) was electrophoretically separated on 0.8% agarose gel and transferred to Hybond (Amersham, Arlington Heights, IL). Blots were hybridized with randomly ^{32}P-labeled IL-5Rα, c-jun, or GAPDH fragments overnight at 42°C in hybridization buffer, washed, and exposed to film as described previously [22].

Results

IL-5Rα expression was previously shown to be upregulated during eosinophilic differentiation of HL-60 clone 15 cells [10; 23]. To investigate whether in HL-60 cells differentiated at pH 7.7 IL-5R expression is also responsive to BA, RNA was isolated from HL-60 7.7 cells treated for various periods with BA. Fig. 1 shows an increase in the expression of the IL-5R by BA treatment within 4 days. Reprobing the blot with a GAPDH probe shows that equal amounts of RNA were loaded in each lane (fig. 1). These results demonstrate that HL-60 7.7 cells are suitable for studying the regulation of the IL-5Rα promoter.

Previously it was shown that the EOS1 enhancer located at -430 to -422 was necessary for the activity of the IL-5Rα chain promoter in HL60 clone 15 cells [12]. We transfected HL-60 7.7BA cells with a number of 5' deletion constructs to determine the IL-5Rα chain promoter region essential for activity in eosinophilic cells. Figure 2A shows that deletion of a promoter region between -467 and -408 results in a complete loss of promoter activity in BA-differentiated HL60 7.7 cells. This region contains the previously identified EOS1 enhancer [12]. However, sequence analysis of the promoter of the IL-5Rα chain revealed the presence of a potential AP-1 binding site (5'-ATGTGTCAG-3'), next to the previously identified EOS1 site [12]. Since members of the

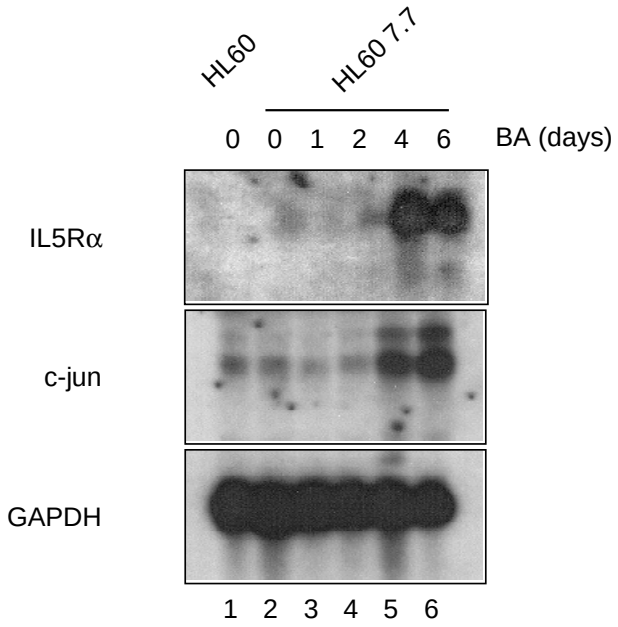

Figure 1. The expression of the IL-5Rα chain is upregulated during the differentiation of HL60 7.7 cells. RNA was isolated from HL60 7.7 cells treated for various times with butyric acid (BA, 0.5 mM) and analyzed for expression of the IL-5Rα chain gene, c-jun and GAPDH. Eosinophilic differentiation of HL60 7.7 cells is clearly accompanied by an increase in expression of the IL-5Rα chain and c-jun genes.

AP-1 family are known as positive regulators of myeloid differentiation (15-17), it is interesting to determine whether the AP-1 site plays a role in promoter activity. Therefore the AP-1 site was mutated in the -467 construct and promoter activity was determined in HL-60 7.7BA cells. A single mutation of the AP-1 site resulted, like a mutation of the EOS1 site, in decreased activity of the construct (fig. 2B). The activity of the construct having both the EOS1 and the AP-1 site mutated was comparable with background level (fig. 2B). These results suggest that the EOS1 and the AP-1 site are both involved in the regulation of the IL-5Rα gene.

To investigate the nature of the proteins binding to the putative AP-1 binding site in the IL-5Rα gene promoter, gel mobility shift assays with a radio-labeled double stranded oligonucleotide containing this AP-1 site were performed. Fig. 3A shows a complex that binds to this AP-1 element in HL-60 7.7BA cells. The specificity of the protein-complex binding to the AP-1 probe was demonstrated by competition with a 10-100-fold molar excess of either the unlabeled cold self-oligonucleotide (fig. 3A). By contrast, oligonucleotides containing a mutated AP-1 site or an SP-1 binding site were not able to compete for binding (fig 3A). To study the properties of the AP-1 element, we examined the competition of this binding with known AP-1 sites. Oligos containing

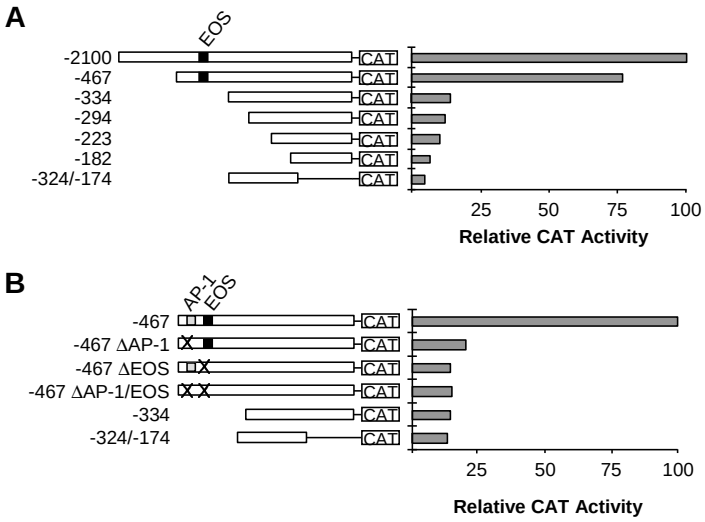

Figure 2. An AP-1 site in the promoter of IL-5Rα chain is involved in its activity in eosinophilic HL60 cells. A HL60 7.7 cells treated for three days with BA were transfected with various progressive 5' deletion constructs of the IL-5R a chain promoter. Two days after transfection, CAT assays were performed to determine the regions of the IL-5Rα chain promoter involved in its eosinophilic activity. **B** IL-5Rα chain promoter constructs with mutations in either the EOS1 site or the AP-1 site were analyzed as described in A. It is clear that both the EOS1 site and the AP-1 site are necessary for full promoter activity.

a TPA (12-*O*-tetradecanoylphorbol-13-acetate) response element (TRE) from the collagenase promoter, or from the c-jun promoter were able to block the binding to the AP-1 site of the IL-5Rα promoter, although to somewhat different extends (fig 3A). In addition, the binding could also blocked by an oligo containing a cAMP response element (CRE) (data not shown). These results indicate that these sites binds proteins or protein-complexes that are also able to bind the AP-1 site of the IL-5Rα promoter.

To further characterize the protein complex binding to the AP-1 binding site within the IL-5Rα promoter, nuclear extracts of HL-60 7.7BA cells were incubated with antibodies against different AP-1 family members prior to detection of DNA-protein interactions by gel mobility shift assay. The antibodies against CREB, CREM and cJun induced a supershift of the labeled DNA probe (fig. 3C), indicating that their respective antigens participate in the formation of the complex bound to the AP-1 site of the IL-5Rα promoter. In contrast, there was no supershift with antibodies against JunD, c-fos, and ATF3 (data not shown).

While CREB and CREM are ubiquitously expressed proteins, the expression of cJun is tightly regulated. Therefore, we analyzed the expression of cJun mRNA in HL-60 7.7 cells after addition of BA which coincides with the increase in expression of the IL-5Rα gene (fig. 1). There is a remarkable increase in the expression of cJun mRNA after four days of addition of BA. Treatment of HL-60 7.7 cells with retinoic acid, an inducer of neutrophilic HL-60 differentiation, does not effect cJun mRNA expression (data not shown). These results indicate that cJun expression is upregulated during eosinophilic differentiation of the HL-60 7.7 cell line, and is likely to play an important role in regulating IL-5Rα expression.

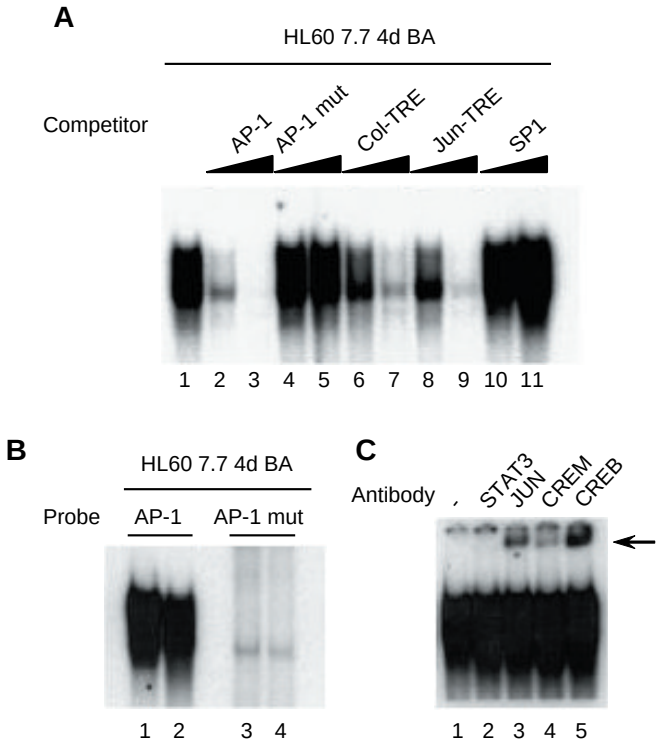

Figure 3. The AP-1 site in the IL-5Rα chain promoter binds multiple AP-1 family proteins. **A** Nuclear extracts isolated from HL60 7.7 cells treated for 4 days with BA were tested for proteins binding to the AP-1 site from the IL-5Rα chain promoter in a gel shift experiment. The specificity of the complex was determined by competition with 10-100 fold molar excess of unlabeled self-oligonucleotide of mutated AP-1 site, SP-1 site, collagenase TRE or c-jun TRE. **B** Mutation of the AP-1 site completely inhibits protein binding to the oligonucleotide, as shown by gel shift analysis using the wild-type or mutated AP-1 site from the IL-5Rα chain promoter as a probe. **C** Supershift analysis with antibodies to c-jun, CREB, CREM show the binding of these proteins to the AP-1 site from the IL-5Rα chain promoter in HL60 7.7BA cells. An antibody to STAT3 was used as a negative control.

Discussion

It has been previously reported that the activity of the IL-5Rα promoter is regulated by the EOS-1 element which binds an unknown eosinophil specific factor [12]. As shown in this study, expression of the IL-5Rα gene is also regulated by the AP-1 element of the promoter. Since single mutation of one of the sites dropped promoter activity, these results indicate that the proteins binding to the AP-1 site and EOS1 site cooperate which suggests that an eosinophil specific transcription factor cooperates with a general transcription factor and together regulate the expression of the IL-5Rα chain.

Gel retardation assays showed the binding of a nuclear protein complex to the AP-1 oligonucleotide. This binding could be inhibited with the unlabeled self-oligonucleotide and also with other known AP-1 elements, such as a CRE site and TRE sites from the collagenase or the c-jun promoter. These results suggest that the AP-1 site of the IL-5Rα promoter is most related to the CRE which can bind dimers of the ATF family and TRE sites which binds cJun. We showed with supershift analysis the presence of the proteins cJun, CREB, and CREM in the shifted complex. There was not a supershift with antibodies against other AP-1 family members such as JunD, c-Fos, and ATF3. This suggests a role for cJun, CREB, and CREM in the binding complexes of the AP-1 site. The expression of cJun mRNA is increased during differentiation of HL-60 cells, indicating that cJun might play a role in eosinophil specific gene transcription. It may be that during eosinophilic differentiation the expression of the IL-5Rα chain gene is regulated by different protein complexes of AP-1 family members. Interestingly, others also found the involvement of AP-1 family members in the differentiation of myeloid cells [15-17].

Recently, a second promoter region for the IL-5Rα gene was identified [24], located within the first intron, between exon 1 and 2. This second promoter is only active in eosinophilic HL-60-C15 cells and the nuclear factor binding to this promoter is also only present in these cells. The existence of another promoter site of the IL-5Rα chain suggests that the transcription of IL-5Rα is under the control of multiple promoters, like other genes such as insulin-like growth factor and colony stimulating factor 1 [25; 26]. It is possible that, during eosinophil maturation, an alternative use of the promoters may determine stage-specific expression of the IL-5Rα gene.

The production of eosinophils from bone marrow progenitors as well as the priming, activation, and enhanced survival of mature eosinophils is regulated by IL-5 [1-4]. Understanding the regulation of the IL-5Rα gene orders an opportunity in controlling the development, differentiation, activation, and prolonged survival of the mature eosinophils. We have shown the contribution of the AP-1 site to this regulation. Further experiments need to be done to resolve the precise roles of the EOS1 and AP-1 elements and binding proteins.

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Chapter 4

A composite C/EBP binding site is essential for the activity of the promoter of the IL-3/ IL-5/ GM-CSF receptor βc gene

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The Journal of Immunology 1999 163: 2674

Abstract

The common β chain (βc) is the main signaling component of the heterodimeric receptors for IL-3, IL-5, and GM-CSF and is primarily expressed on myeloid cells. The proximal βc promoter is regulated by GGAA-binding proteins, including PU.1, a hematopoietic specific member of the Ets family. However, it is not likely that PU.1 alone accounts for the myeloid-restricted expression of the βc subunit. Here we describe the identification of a C/EBP-binding enhancer that is located 2 kb upstream of the transcription start site. The enhancer contains two elements that bind C/EBPα and -β in U937 cells, while also C/EBPε is also bound in extracts of HL-60 cells. Importantly, deletion of the enhancer or mutation of either of one of the C/EBP sites results in a complete loss of promoter activity in cell lines as well as in primary cells, showing the importance of C/EBP members in βc gene activation. We further show that PU.1 has to cooperate with C/EBP proteins to induce βc transcription. Since the βc is already expressed on CD34+ cells, these results demonstrate that both C/EBP and PU.1 are not only important for the myeloid-specific gene regulation at later stages of myeloid differentiation.

Introduction

Hematopoiesis is a developmental process in which pluripotent hematopoietic stem cells proliferate and differentiate to lineage-committed progenitors which eventually generate all kinds of mature, terminally differentiated blood cells [1]. The multistep processes leading to the differentiation of CD34+ stem cells toward the erythroid, lymphoid, or myeloid lineages are, at least in part regulated by a network of cytokines. Of these, G-CSF, M-CSF, GM-CSF, IL-3, and IL-5 are implicated in the development of myeloid cells, which include monocytes/macrophages and the neutrophilic-, eosinophilic-, and basophilic granulocytes [2]. Where IL-3 and GM-CSF act on early progenitors, IL-5, G-CSF, and M-CSF stimulate the late differentiation of eosinophilic, neutrophilic, and monocytic lineages, respectively [2-4].

Although IL-5, IL-3, and GM-CSF all have distinct effects on different target cells, they elicit similar responses in cells responsive to all three cytokines and they even cross-compete for binding to the same cell [5,6]. Molecular cloning of the receptor components and reconstitution of the functional receptors have revealed that the receptors for the three cytokines are heterodimers composed of a cytokine-specific α chain (IL-3Rα, IL-5Rα, and GM-CSFRα) and a common signaling β chain (βc) [7-9]. Both the α and β chains are glycosylated proteins and members of the class I cytokine receptor family [10]. Although the human βc subunit has no binding capacity by itself, it associates with the low affinity α chains to form a high affinity receptor [11-13]. Besides its role in high affinity binding, the βc chain plays a major role in IL-3, IL-5, and GM-CSF mediated signal transduction, explaining the functional overlap of these cytokines [14]. Interestingly, the mouse has two β subunits, known as βc (AIC2B) and βIL-3 (AIC2A) [15,16]. The mouse βc chain is the common β

subunit for the mouse IL-3, IL-5, and GM-CSF receptors, in analogy with the human βc. Although βc and βIL-3 have 91% homology at the amino acid level, βIL-3 binds IL-3 with low affinity and does not form a high affinity receptor with IL-5Rα and GM-CSFRα, in contradiction to βc [17]. We previously reported the cloning of the human βc gene and the remainders of a βc pseudogene, suggesting a loss of βIL-3 in the human rather than murine-specific gene duplication [18].

In both the human and murine systems, binding of IL-3/IL-5/GM-CSF induces receptor multimerization, most likely in a ligand:α:β stoichiometry of 2:2:2 [19]. This leads to the activation of JAK2, a protein tyrosine kinase that is constitutively associated with the membrane-proximal region of the βc chain [20,21]. Subsequently, signaling molecules of several signal transduction pathways are activated, including STAT5 [22,23], p21Ras [24], ERK1/2 [25], JNK/SAPK [26], p38 [27], and PI3K [28,29](for a review, see [30]). Because the βc subunit is involved in IL-3, IL-5, and GM-CSF signaling, expression of the βc chain is important for all myeloid cells and their precursors. Therefore, elucidating the factors that govern its expression will help to understand the events that regulate myeloid-lineage commitment.

The βc chain is already expressed on hematopoietic CD34+ stem cells [31], while at later stages of differentiation expression of the βc chain is mainly restricted to the myeloid lineages [31]. Uncovering the molecular mechanisms that drive its expression will contribute to understanding of the events of myeloid commitment and differentiation. In the last few years it has become clear that PU.1, a hematopoietic-specific member of the Ets family, and the C/EBP family are key transcription factors in myeloid-specific gene activation [32]. Receptors that play a significant role in myeloid development, such as the GM-CSFRα, G-CSFR, and the M-CSFR, have all been shown to be regulated by the combination of PU.1 and C/EBP proteins [33-35]. Previous studies described the isolation of the genes for the human and murine βc/βIL-3 subunits [18, 36]. We have shown that PU.1 and another, unidentified GGAA-binding protein activate the human βc gene by binding to the proximal promoter region (at -65bp and -45bp relative to the transcription start site, respectively) [18]. PU.1, a hematopoietic specific member of the Ets transcription factor family [37], is expressed in myeloid and B cells and has been shown to regulate a number of myeloid specific genes, including the M-CSFR [33], G-CSFR [34], GM-CSFRα [35], eosinophil-derived neurotoxin [38], myeloperoxidase [39], and PU.1 itself [40]. Other factors that have been shown to be important for myeloid-specific promoter regulation are members of the CCAAT/enhancer binding protein (C/EBP) family. Members of the C/EBP family have been shown to have a changing temporal expression pattern during myeloid differentiation. C/EBPα is expressed at high levels in early progenitors, C/EBPβ and -δ become more prominent at later stages, while C/EBPε is only expressed during granulopoiesis [41-43]. C/EBPα, a basic-region leucine zipper transcription factor, originally characterized in liver and adipose tissue [44], is expressed in early myeloid precursors [41] and has also been shown to be important for the expression of the M-CSFR, G-CSFR, and GM-CSFRα [33-35]. Another C/EBP member that is important for myeloid gene expression is C/EBPε, which was recently described to be strongly induced during granulocytic differentiation

[42,43]. Gene targeting of both C/EBPα and C/EBPε results in impaired granulopoiesis [45,46], clearly demonstrating their roles in myeloid development.

Here we show that, analogous to the M-CSFR, G-CSFR, and GM-CSFRα, the βc is regulated by C/EBP members. We identified a crucial enhancer located 2 kb upstream of the transcription start site that contains two C/EBP-binding motifs. Both sites are able to bind C/EBPα, -β, and -ε, while mutation of the sites results in a complete loss of promoter activity.

Materials and Methods

Cells, cDNAs, oligonucleotides, and antibodies

COS-1 cells were cultured in Dulbecco's modifies Eagle medium (DMEM; Life Technologies, Gaithersburg, MD) supplemented with 8% heat inactivated fetal calf serum (FCS; Life Technologies). The monocytic cell line U937 and the promyelocytic leukemia cell line HL-60 were maintained in RPMI 1640 (Life Technologies) supplemented with 8% FetalClone I (HyClone, Logan, UT).

$CD34^+$ cells were isolated from cord blood and differentiated as described by Caldenhoven *et al*. [47]. In short, mononuclear cells were obtained by centrifugation of the blood on a Ficoll-Hypaque gradient (density 1.077 g/ml). Mononuclear cells were incubated with $CD34^+$ antibody and magnetic beads, prior to positive selection on a column that was placed in a magnetic field. Cells were cultured in Iscove's modified Dulbecco medium (IMDM; Life Technologies) supplemented with 10% FCS, Stem Cell Factor (100 ng/ml), FLT-3 ligand (100 ng/ml), IL-3 (0.1nmol/l), GM-CSF (0.1nmol/l), and IL-5 (0.1nmol/l). After three days, cells were cultured in IMDM supplemented with 10% FCS, IL-3 and IL-5. From day 21, IL-3 was also omitted from the medium. Viability remained >95% throughout the 28-day experiment.

Human C/EBPα (a kind gift from Dr. G.W.M. Swart, University of Nijmegen, The Netherlands), hC/EBPβ (a kind gift from Prof. S. Akira, Hyogo College of Medicine, Nishinomoya, Japan), hC/EBPγ (cloned by PCR from HL-60 cDNA), rat C/EBPδ (a kind gift from Prof. S.L. McKnight, John Hopkins University, Baltimore, MD), hC/EBPε (cloned by PCR from HL-60 cDNA), and PU.1 (a kind gift from Prof. R.M. Maki, Burnham Institute, La Jolla, CA) were cloned into the expression vector pLNCX (Clontech, Palo Alto, CA).

The following oligonucleotides were used in this study: for PCR cloning of C/EBPγ, γF (5'-TGGCAAGGGAGAGTGCCCAA-3') and γR (5'-TGAGGTCTACTG-TCCTGCAT-3'); for cloning of C/EBPε, εF (5'-TCAAGAGCAGTGGGGGCGGG-3') and εR (5'-TCCACCAGCCAGCCTCAGCT-3'); for site-directed mutagenesis of C/EBP site 1, ENHC1 mut (5'-AACTCAAAGGGGGG GCCCACATGAAGGGTA-3'); for C/EBP-site 2, ENHC2 mut (5'-CTCTGTATTTTGCTCGA GTTTCAGAATAAA-3'); andfor band-shift analysis (only upper strand is shown), C1 wt (5'-AGCTTCAAAGGGGGTTTC-CACATGAAGGGA-3'), C1 mut (5'-AGCTTCAAAGGGGGGGC CCACATGAAGGGA-3'), C2 wt (5'-AGCTTGCTCTGTATTTTGAAATAGTTTCAA-3'), C2 mut

(5'-AGCTTG-CTCTGTATTTTGCTCGAGTTTCAA-3'), and -65 (5'-AGCTTCCGGCACTGC-TTCCTCTTTCTGCTA-3').

The following antibodies were used in this study: anti-C/EBPα (rabbit polyclonal IgG 14AA, sc-061X; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-C/EBPβ (rabbit polyclonal IgG C-19, sc-150X; Santa Cruz Biotechnologies), anti-C/EBPδ (rabbit polyclonal IgG M-17, sc-636X; Santa Cruz Biotechnologies), anti-C/EBPε (rabbit polyclonal IgG C-22, sc-158X; Santa Cruz Biotechnologies), and anti-Fli-1 (rabbit polyclonal IgG C-19, sc-356X; Santa Cruz Biotechnologies).

Plasmids for promoter analysis, transient transfection, and CAT assay

The 2.7-kb *Hin*dIII fragment of the βc subunit promoter (-2096/+600) was cloned into the *Hin*dIII site of the promoterless pBLCAT3 vector [48]. Deletion construct -1967/+600CAT3 was generated by cloning the *Hin*cII- *Hin*dIII fragment (-1967/+600) into *Xba*I and *Xho*I sites of pBLCAT3 via the *Sma*I- *Hin*dIII sites of SK- (Stratagene). The 129 bp *Hin*dIII -*Hin*cII fragment (-2096 to -1967) was cloned into the *Hin*dIII and *Bam*HI sites of pBLCAT2 [48] and the *Hin*dIII and *Pst*I sites of TATACAT [49] to generate HH129CAT2 and HH129TATA, respectively, via the *Hin*dIII and *Sma*I sites of a modified pSG5 (Stratagene). The *Bgl*II site at -289 was used to generate -2096/-289CAT2 and -1967/-289CAT2. Site directed mutagenesis was performed according to the method of Kunkel [50].

For transfection experiments, COS-1 cells were cultured in six-well dishes (Nunc, Copenhagen, Denmark), and 3 h later, the cells were transfected with 6 μg of supercoiled plasmid DNA as described previously [51]. Following 16 to 20 h exposure to the calcium-phosphate precipitate, medium was refreshed. Cells were harvested for CAT assays 24 h later. Transfection of U937, HL-60, eosinophils and neutrophils was performed by electroporation at 300V and 960 μF (10^7 cells, 20 μg of supercoiled plasmid DNA). In co-transfection experiments 10 μg of promoter construct was transfected with 10 μg of cDNA. Two days after transfection cells were harvested for CAT assays. CAT assays were performed as follows. Cells were lysed by repeated freeze-thawing in 250 mM Tris (pH7.4) and 25 mM ethylenediaminetetraacetic acid (EDTA). Cellular extract was then incubated in a total volume of 200 μl containing 250 mM Tris (pH7.4), 2% glycerol, 0.3 mM Butyryl Coenzyme A (Sigma, St. Louis, MO), and 0.05 μCi of [^{14}C] Chloroamphenicol (Amersham, Arlington Heights, IL) for 2 h at 37°C. Reaction products were then extracted using 400 μl of xylene/pristane (1:2), and the percentage of acetylated products was then determined using liquid scintillation counting. All experiments were performed at least four times. A *LacZ* reporter was used to correct for transfection efficiency.

Gel retardation assay

Nuclear extracts were prepared from U937 and HL-60 cells following a previously described procedure [52]. Oligonucleotide probes were labeled by filling in the cohesive ends with [α-^{32}P]dCTP using Klenow fragment of DNA polymerase I. Gel retardation assays were performed as follows.

Nuclear extracts (10 μg) were incubated in a final volume of 30 μL containing 10 mM Tris (pH7.4), 10% glycerol, 2 mM EDTA, 50 mM NaCl, 0.05% Nonidet P-40, 300 μg/ml bovine serum albumin (BSA), 30μg/ml poly(dI/dC), 0.5 mM dithiothreitol (DTT), and 1.0 ng of ^{32}P-labeled probe for 20 min at room temperature. Complexes were then separated through non-denaturating 5% polyacrylamide gels and visualized by autoradiography. In competition experiments, a 10 to 100-fold molar excess of wild type or mutated oligonucleotide was added to the reaction mix for 5 min before addition of the labeled probe. For supershift analysis, nuclear extracts were pre-incubated with 2μg antibody for 40 min on ice before the addition of the labeled probe.

Results

A 2-kb upstream enhancer regulates βc subunit expression

In a previous study we reported the isolation of a 2.7-kb *Hin*dIII fragment, which contains the 44 bp exon 1, 2096 bp of upstream sequences, and 598 bp of the first intron of the human βc subunit

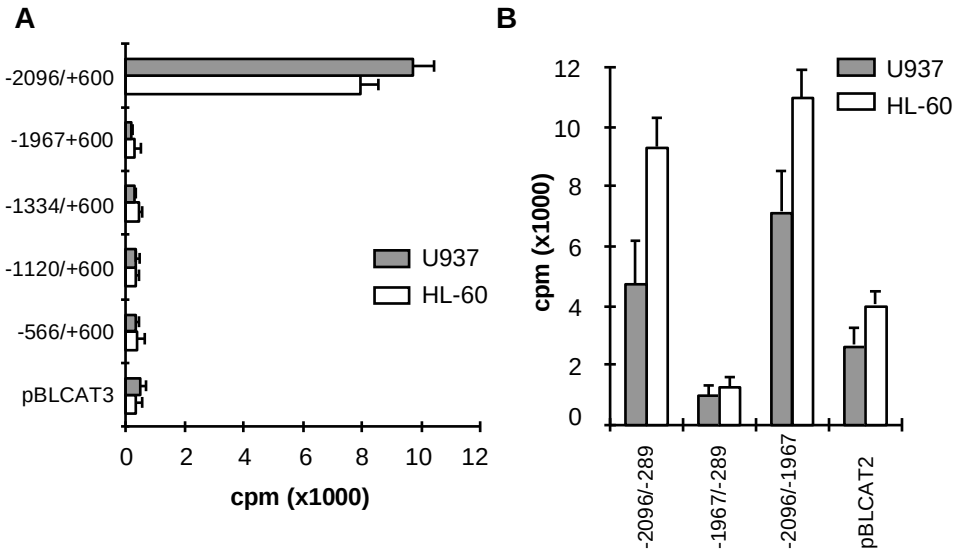

Figure 1. An upstream enhancer is crucial for βc promoter activity. A. U937 and HL-60 cells were transfected with successive 5' deletion constructs of the βc promoter cloned in the promoterless pBLCAT3 reporter. Two days after transfection CAT activity was determined. Construct -2096/+600CAT3, which is active in both cell types, contains the 44 bp exon 1, 2096 bp of upstream sequences and 598 bp of the first intron. Deletion of 129 bp at the 5' end results in the complete loss of promoter activity. **B.** Constructs that lack the proximal promoter, with (-2096/-289), without (-1967/-289) the enhancer, and the isolated enhancer (-2096/-1967) were cloned into the pBLCAT2 vector, which contains the minimal thymidine kinase promoter. Deletion of the enhancer element again results in the loss of promoter activity, while the isolated enhancer element has maximal CAT activity. This figure demonstrates that the fragment from -2096 to -1967 bp contains a functionally important enhancer element. Bars indicate the mean value of at least three independent experiments; standard deviation is indicated by error bars.

gene [18]. After cloning into the promoterless pBLCAT3 reporter plasmid (-2096/+600CAT3), transfection experiments in monocytic U937 cells and promyelocytic leukemia HL-60 cells showed that this fragment contains a functionally active promoter. To localize *cis*-activating elements, successive 5' deletion constructs were generated. The *Nde*I (at -566), *Eco*RI (at -1120), and *Sma*I (at -1334) sites were used to generate -566/+600CAT3, -1120/+600CAT3, and -1334/+600CAT3, respectively. As shown in figure 1A, these constructs completely lost their activity. Also construct -1967/+600CAT3, made by using the *Hin*cII site at -1967, has no promoter activity, suggesting that an important positively regulating element has to be present between -2096bp and -1967bp. To test this hypothesis, the 129bp *Hin*dIII -*Hin*cII fragment, as well as the fragments from -2096 to -289bp and from -1967 to -289bp were cloned into the pBLCAT2 vector, a reporter plasmid containing the minimal thymidine kinase promoter [48]. The generated constructs -2096/-1967CAT2, -2096/-289CAT2, and -1967/-289CAT2 were then transfected into U937 cells and HL-60 cells and tested for promoter activity. Figure 1B shows that -2096/-289CAT2 is active in both cell types, while -1967/-289CAT2 has lost this capacity. Indeed, -2096/-1967CAT2 is highly active in both cell types, clearly demonstrating the presence of an enhancer between -2096bp and -1967bp.

The enhancer contains two C/EBP binding motifs

To localize potential transcription factor binding sites, the 129 bp enhancer sequence (fig 2A) was subjected to the TFMATRIX transcription factor database (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). As shown in figure 2A, one NFκB and two C/EBP binding sites were identified. Because C/EBP family members play an important role in the constitutive and inducible expression of a number myeloid-specific genes, the putative C/EBP sites (C1 and C2, fg 2A) were studied in further detail. Band shift analysis was used to investigate whether C1 and C2 were protein binding elements. Oligonucleotide probes, each spanning a single C/EBP site, were end labeled and incubated with 10 μg of U937 and HL-60 nuclear extract. Figure 2B shows that elements C1 and C2 are both able to bind a partially overlapping set of protein complexes (lanes 1 and 8). The binding of most complexes could be competed by addition of a 10 to 100-fold molar excess of cold self oligo (lanes 2-3 and 9-10), but not with mutant C1 (lanes 4-5), mutant C2 (lanes 11-12), or non-related -65 oligo (lanes 6-7 and lanes 13-14), a sequence that specifically binds transcription factor PU.1 in HL-60 and U937 cells. One complex (indicated by an arrow) can also be competed with the mutated or non-related oligos. These results demonstrate the specificity of the complexes binding to elements C1 and C2, except for the indicated complex. To further characterize the proteins binding to C1 and C2, the nuclear extracts were pre-incubated with specific antibodies against C/EBP members and Fli-1, a member of the Ets family. In HL-60 cells, supershifted complexes appear when extracts are incubated with anti-C/EBPα, -β, and -ε (figure 2c, lanes 2, 3, and 5 for C1, lanes 8, 9, and 11 for C2), but not with anti-C/EBPδ (lanes 4 and 10) and anti-Fli-1 control antibody (lanes 6 and 12). Pre-incubation with anti-C/EBPδ (lanes 4 and 10) results in the disappearance of a complex (indicated by an arrow). It is not clear whether

this complex is C/EBPδ, because competition experiments (see above) suggested that this is a nonspecific complex. Almost identical results were obtained with U937 cells, but no supershift was observed when extracts were incubated with anti-C/EBPε (data not shown). Taken together, these results show that different C/EBP proteins, including C/EBPα, -β, and -ε can bind to two distinct elements in the upstream enhancer.

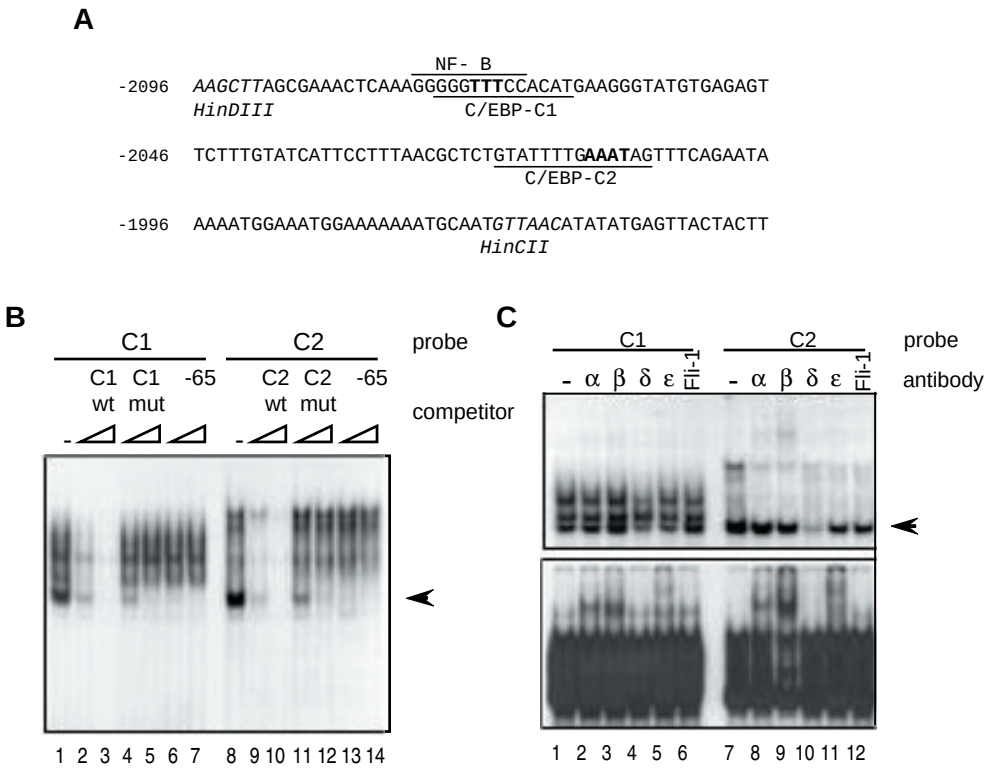

Figure 2. Two elements in the enhancer bind C/EBP-containing complexes. A. The 150 bp nucleotide sequence of the 5' end of construct -2096/+600. The 129 bp *Hin*dIII/*Hin*CII fragment was subjected to a transcription factor database; one NFκB site and two C/EBP binding elements (C1 and C2, underlined) were identified. Nucleotides which were mutated to inhibit C/EBP binding are indicated in bold face. **B.** Nuclear extracts of HL-60 cells were incubated with oligonucleotides spanning a single C/EBP site and analyzed in a band shift assay. Elements C1 and C2 bind a partially overlapping set of complexes (lanes 1 and 8), which can be competed with a 10 to 100 fold molar excess of cold self oligo (lanes 2, 3 and 9, 10). When mutant (C1mut and C2mut) or non-related oligo (-65, a PU.1 binding element) is used, only one complex is competed for binding (indicated by the arrow). **C.** Before addition of the labeled oligo, 2 μg of indicated antibody was added to nuclear extracts of HL-60 cells. Pre-incubation with antibodies against C/EBPα, -β, and -ε, but not with control antibody against Fli-1 results in the appearance of supershifted complexes (lower panel). The upper panel is a shorter exposure of the same gel. The arrow indicates the complex which disappears when anti-C/EBPδ is added. It is unclear whether this complex contains C/EBPδ, since figure 2A suggests that this is a non-specific complex. This figure shows that the enhancer contains two discrete elements that specifically bind C/EBPα, -β, and -ε in HL-60 nuclear extracts. In U937 cells similar results were obtained, only no binding of C/EBPε was detected.

The C/EBP sites are essential for enhancer activity

To elucidate whether the C/EBP binding elements are functionally important, both C1 and C2 were mutated in the -2096/-1967CAT2 construct and tested for promoter activity in U937 and HL-60 cells. Single mutation of either C1 or C2 already reduced promoter activity to the background level (fig 3A), as occurs when both sites are mutated. These data show that both elements are essential for enhancer activity, and suggest the cooperation of the proteins binding to the elements. Next we co-transfected the wild type and mutant constructs with the cDNAs of several C/EBP family members. Probably due to a cryptic C/EBP site in the pBLCAT2 vector, co-transfection of C/EBP cDNAs with pBLCAT2 resulted in moderately enhanced background values (unpublished result). Therefore the enhancer constructs were cloned into the pLT-G plasmid, a CAT-vector with a synthetic TATA-box. Figure 3b shows that C/EBPα, -β, -δ, and -ε induced a 4-5 fold induction of CAT activity when co-transfected with the wild type -2096/-1967 construct in HL-60 cells (similar results were obtained in U937 cells, not shown). When C/EBP elements C1 and C2 were mutated, the effect of C/EBP co-transfection was dramatically decreased. The transactivating capacity of C/EBPδ is of interest, because it is unclear whether this protein binds to the enhancer in a band shift assay (see above). Nevertheless, C/EBPδ binds to C1 and C2 when overexpressed in COS cells (data not shown).

To further demonstrate the importance of C/EBPs for enhancer activity, the -2096/-1967CAT2 construct was also co-transfected with C/EBPγ, a dominant negative C/EBP member [53], and with an anti-sense construct of C/EBPα. As shown in figure 3c, co-transfection of anti-sense C/EBPα reduces promoter activity with 65% in U937 cells and with 50% in HL-60 cells, while C/EBPγ reduces promoter activity with 70% in both cell types. These results clearly demonstrate the role of C/EBP proteins in the regulation of the βc chain gene.

To demonstrate that the C/EBP sites also contribute to βc promoter activity in primary cells as opposed to tumor cell lines, we transfected the C1 and C2 double mutant in the -2096/-1967CAT2 construct into in vitro differentiated eosinophils and neutrophils (see Materials and Methods). As we found for HL-60 and U937 cells (see Fig. 3A), mutation of the C/EBP sites significantly reduces enhancer activity in both eosinophils and neutrophil (Fig. 3D). This clearly shows that these C/EBP sites are involved in βc promoter activity in primary cells.

C/EBP and PU.1 cooperate to activate the βc promoter in non-hematopoietic cells

We have previously shown that binding of PU.1 to a GGAA element at -65 bp relative to the transcription start site is crucial for βc chain expression. To more precisely elucidate the roles of PU.1 and C/EBP in βc chain expression, these factors were co-transfected with different promoter constructs in COS fibroblasts. Figure 4A shows that both PU.1 and C/EBPα enhanced the promoter activity of the full promoter construct -2096/+600CAT3 by about 10 fold. However, co-transfection of C/EBPα together with PU.1 resulted in a 30 fold increase in promoter activity. As expected, C/EBPα could no longer activate transcription when the C/EBP enhancer was deleted (construct -1967/+600CAT3, figure 4A). Surprisingly, also PU.1 could no longer activate this

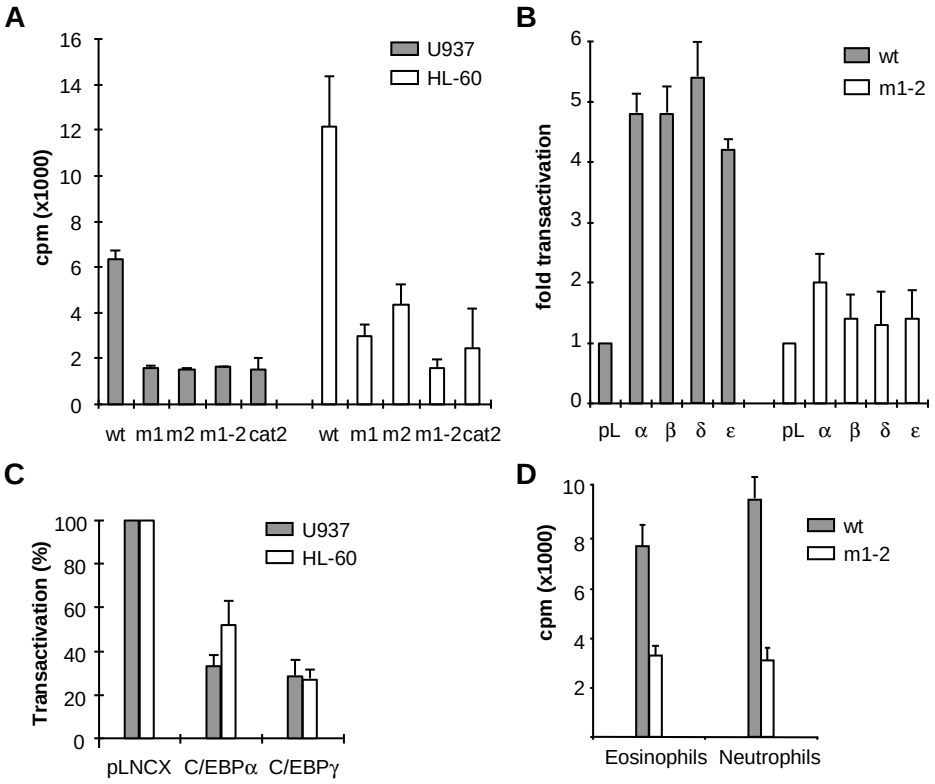

Figure 3. Enhancer activity is mediated via the C/EBP binding sites. A. C/EBP elements C1and C2 were mutated alone (m1 and m2, respectively) or both (m1-2) in the -2096/-1967CAT2 construct and tested for activity in a CAT assay in both U937 and HL-60 cells. Single mutation of either of one of the sites reduces CAT activity to background level in both cell types. **B.** Co-transfection in HL-60 cells of the wild type -2096/-1967 construct with cDNAs encoding C/EBPα , -β, -δ, and -ε results in 4-5 times increase of CAT activity, when compared to empty pLNCX (pL) vector. The double mutant is not responsive to C/EBP co-transfection. For this experiment the constructs were cloned into the pLT-G reporter, since C/EBP co-transfection has a moderate effect on empty pBLCAT2 vector. Similar results were obtained in U937 cells. **C.** Co-transfection of C/EBPγ, a C/EBP member that lacks the transactivation domain or anti-sense C/EBPα significantly reduces promoter activity of -2096/-1967CAT2 in both U937 and HL-60 cells. This fg ure shows that the C/EBP elements are responsive to C/EBPα to -ε and are both crucial in promoter activation. Bars indicate the mean value of at least three independent experiments; standard deviation is indicated by error bars.

construct. This was not due to a PU.1 binding site in the -2096/-1967 sequence, because figure 4B shows that co-transfection of PU.1 did not affect the activity of construct -2096/-1967CAT2, which is in contrast to co-transfection of C/EBPα. Construct -226/+33CAT3, which contains only the promoter proximal region, can only be induced by PU.1, not by C/EBPα (figure 4B, second panel). If trans-activation of the full promoter construct (-2096/+600CAT3) by PU.I is dependent on proteins binding to the C/EBP sites, this would suggest that COS cells contain endogenous binding activity for the C/EBP sites. Therefore, we performed band shift experiments with COS

nuclear extracts. Indeed, binding site C2 binds a specific protein complex in COS cells, that can be competed with access unlabeled probe, but not with a mutated C2 sequence or an unrelated oligonucleotide (Fig. 4C). We did not observe specific binding activity for C1 in COS cells (data not shown). Taken together, these results indicate that PU.1 and C/EBP functionally cooperate to enhance βc expression, although their respective binding elements are not clustered as is described for the GM-CSFRα, G-CSFR, and M-CSFR promoters.

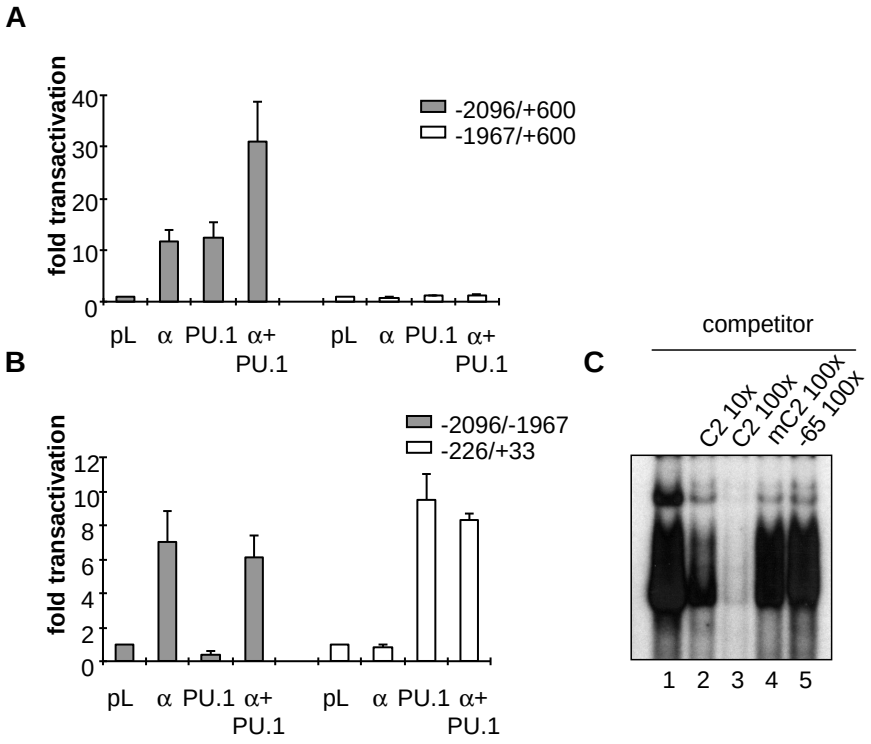

Figure 4. C/EBP and PU.1 cooperate in βc gene transcription. A. C/EBPα and PU.1 were co-transfected with different promoter constructs in COS fibroblasts by the calcium phosphate precipitation method. C/EBPα and PU.1 each induce a 10 fold transactivation when co-transfected with the full promoter construct -2096/+600CAT3. When both factors are transfected a 30-fold induction is measured, indicating that these proteins cooperate in βc gene activation. The effect of both C/EBP and PU.1 overexpression is completely lost when the C/EBP-binding enhancer is deleted (-1967/+600CAT3). **B.** COS fibroblasts were transfected with different promoter constructs, C/EBPα and PU.1 by the calcium phosphate precipitation method. The upstream enhancer (-2096/-1967) does not contain a functional PU.1 site, while activity of the proximal promoter region (-226/+33) can be enhanced by PU.1, but not C/EBPα co-transfection. These results demonstrate that PU.1, binding to the promoter proximal region, can no longer activate the βc promoter in the absence of C/EBP in the context of the full promoter. Bars indicate the mean value of at least three independent experiments; standard deviation is indicated by error bars. **C.** Nuclear extracts of COS cells were incubated with an oligonucleotide spanning C/EBP site C2 and analyzed in a band shift assay. Element C2 binds a set of complexes, which can be competed with a 10 to 100 fold molar excess of cold self oligo. When mutant (C2mut) or non-related oligo (-65, a PU.1 binding element) is used, no competition was observed.

Discussion

Members of the C/EBP family have been shown to have a changing temporal expression pattern during myeloid differentiation. C/EBPα is expressed at high levels in early progenitors, C/EBPβ and -δ become more prominent at later stages, while C/EBPε is only expressed during granulopoiesis [41-43]. Alternative splicing and the ability of all members of the C/EBP family to form homo- and heterodimers leads to a variety of possible DNA-binding complexes. It is likely that these complexes have specific affinities for particular binding sites and that this mechanism plays an important role in the specific regulation of certain genes by C/EBPs. In the βc subunit enhancer we identified two different binding sites that bind multiple C/EBP containing complexes (figures 2A and 2B). Both elements bind C/EBPα, -β, and -ε in HL-60, while in U937 cells, which express C/EBPε at a very low level, only binding of C/EBPα and -β was observed. Binding of C/EBPδ is unclear. In a band shift assay, addition of anti-C/EBPδ antibody results in the disappearance of a specific DNA-binding complex. However, the same complex can be competed with mutant and non-related oligonucleotides, suggesting that this is a non-specific binding complex. On the other hand, C/EBPδ from transfected COS cells can bind both sites and C/EBPδ can transactivate the βc promoter in co-transfection experiments (data not shown; figure 3B). Experiments with murine embryonic stem cells and blastocysts have shown that transcription of βc and βIL-3 is induced at the same time as the CD34 gene [54]. In CD34+ cells, the C/EBPα is the predominant expressed isoform [55] and, therefore, is likely to be involved in the initial induction of the βc subunit gene(s). Nevertheless, expression of βc/βIL-3 is unaffected in C/EBPα deficient mice, while expression of the G-CSFR is completely blocked [45]. This might indicate that C/EBP members can compensate for each other in some, but not all, situations.

We have shown that both sites can bind C/EBP independently and that both sites are functional. Mutation of either site results in almost a complete loss of promoter activity in cell lines as well as in primary eosinophils and neutrophils (figure 3A and 3D), suggesting that both sites are crucial for transcription activation. However, it is not clear whether both sites are occupied by C/EBP simultaneously *in vivo*. Transcription could be reduced by co-transfection of C/EBPγ, a dominant negative C/EBP member, and anti sense C/EBPα, further demonstrating the specific binding of C/EBP proteins to the enhancer sequence (figure 3C).

(Co)transfection experiments with different deletion constructs showed the cooperation of C/EBP proteins and PU.1 in the regulation of the βc gene (figure 4), reinforcing the significance of these transcription factors in myeloid development. The combination of C/EBPs and PU.1 is also important for G-CSFR, GM-CSFRα, and M-CSFR expression. Functional elements for both proteins have been identified in the proximal promoters of these genes. The sites for PU.1 and C/EBP are separated by 15 bp (GM-CSFRα), 35 bp (M-CSFR), or 85 bp (G-CSFR), indicating that both proteins are likely to interact. Furthermore, both elements are close to the transcription start site (<100 bp) [33-35]. In the βc subunit promoter the situation is different. Two functional GGAA-elements are present in the proximal promoter region, while important C/EBP binding sequences

are located 2 kb upstream. When isolated, the PU.1-binding proximal promoter sequence has a high activity (construct -226/+33, figure 4). Interestingly, in the full promoter context (construct -2096/+600), this activity is dependent on the C/EBP binding enhancer, because deletion of the enhancer (construct -1967/+600) results in the complete loss of promoter activity. This again suggests an interaction between C/EBPs and PU.1. Why binding of PU.1 is not enough to induce promoter activity of construct -1967/+600 is not known. Initial experiments opened the possibility that additional, yet unidentified proteins play a role in βc gene activation. It is possible that this factor(s), PU.1 and C/EBP all have to be present to obtain a transcriptionally active complex, while in the absence of C/EBP the additional protein blocks transcription by PU.1. Although future experiments are necessary to identify possible additional promoter elements and/or proteins that are involved in βc subunit expression, we have shown that both PU.1 and C/EBP play a critical role in βc chain transcription.

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Chapter 5

C/EBP transcription factors control eosinophil differentiation and proliferation from human umbilical cord blood derived CD34+ cells

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submitted

Abstract

We have investigated the role of C/EBP transcription factors during the IL-5 induced differentiation of human CD34+ cells towards eosinophils. For this purpose we used human umbilical cord blood derived CD34+ cells and cultured them in the presence IL-5. In this *ex vivo* system C/EBPα is expressed at high levels during the induction phase of differentiation and is down-regulated with maturation. C/EBPε on the other hand, is induced during differentiation of CD34+ cells and remains expressed at a high level until terminal differentiation. In order to establish whether C/EBP proteins play a role in eosinophil differentiation, retroviral transductions were performed to ectopically express a dominant negative version of the C/EBP proteins. Transduction of CD34+ cells with DN-C/EBP results in an inhibition of proliferation and in a block of differentiation as shown by morphology. Proliferation decreased from a thirty times fold induction to a ten times fold induction. Using this *ex vivo* system of eosinophil differentiation we were able to induce seventy-five percent juvenile eosinophils. On the other hand in cells transduced with DN-C/EBP remain in the blast stage and only four percent juvenile eosinophils could be detected. RNA of the common β chain of the IL-3/IL-5 and GM-CSF receptor was strongly decreased in cells ectopically expressing DN-C/EBP. These results show that C/EBP transcription factors are involved in both the proliferation and differentiation of human eosinophils. The control of the βc promoter provides a mechanism of how differentiation is regulated by C/EBP factors.

Introduction

Eosinophils are major effector cells in allergic diseases, such as allergic asthma [1; 2]. They are recruited to and activated in lung tissue where they participate in processes that lead to tissue damage characteristic for the pathophysiology of allergic asthma [3-5]. The pro-inflammatory effects of eosinophils are mediated by the release of cytokines, chemokines and lipid mediators, such as TNF-α, IL-8, PAF, and LTC_4 [6; 7]. Cytotoxic effects are provoked by reactive oxygen species produced by a membrane bound NADPH oxidase and by toxic basic proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) [8-10].

Eosinophils are derived from pluripotent hematopoietic stem cells (HSC) in the bone marrow[11]. These HSC's are responsible for the maintenance of adequate numbers of terminally differentiated hematopoietic cells during health and disease [12]. The HSC mature into fully differentiated eosinophils via the granulocyte/ erythrocyte/ macrophage/ megakaryocyte-colony forming unit (GEMM-CFU) and eosinophil/basophil-colony forming unit (EO/B-CFU) [13-16]. Tight regulation of expression of cytokines, colony stimulating factors, their receptors, and transcription factors coordinate lineage specific differentiation. Interleukin-3 (IL-3), granulocyte macrophage

colony stimulating factor (GM-CSF), and IL-5 have been shown to play a major role in the differentiation of eosinophils [17-19]. Development of hematopoietic cells is controlled by lineage-specific transcription factors. CAATT/enhancer binding protein (C/EBP), PU.1, and c-myb have emerged as key regulators of gene expression during granulopoiesis [20-22].

The C/EBP family of transcription factors belongs to the basic region/leucine zipper (bZIP) transcription factors. The C/EBP proteins share structural and functional features and to date, six members have been cloned and characterized: C/EBPα, β, γ, δ, ε, and ζ [23-27]. C/EBP proteins contain a carboxy-terminal dimerisation domain (leucine-zipper), a DNA-binding domain (basic-region), and an N-terminal activation domain [27]. C/EBPs can form homodimers and heterodimers with other C/EBP proteins and with other transcription factors via their leucine zipper region [27]. In contrast to the other C/EBP members, which are widely expressed, C/EBPε is predominantly expressed at high levels in the late stages of granulopoiesis and in T-lymphocytes [28-30]. The C/EBP family members are known to exert pleiotropic effects in the tissues in which they are expressed [30]. This effect may be due to their tissue and differentiation-stage specific expression, their ability to dimerise with members of their own family of transcription factors, and their capability to interact with other transcription factors such as PU.1 and Fos/Jun.

C/EBPα has been shown to be expressed in early myeloid cells and to be involved in the regulation of a number of granulocyte-specific genes [20; 31-37]. Mice deficient in C/EBPα display an early block in the maturation of granulocytes [38]. Both C/EBPβ and C/EBPδ play a role in mediating the inflammatory responses [39]. Recently C/EBPβ was described to play a role in the regulation of MBP [40]. C/EBPε is essential for terminal differentiation and functional maturation of committed granulocyte progenitor cells. As a consequence of its characterization, using both in vitro and knockout mouse models, C/EBPε has been identified as a critical regulator of terminal granulopoiesis and one of the causative mutations in a severe human disease, neutrophil-specific granule deficiency [41].

In the present study, we have investigated the role of C/EBP transcription factors during the differentiation of human $CD34^+$ cells towards eosinophils in the presence of IL-5. Our results demonstrate that C/EBPα is highly expressed at the beginning of eosinophil differentiation and is down-regulated during maturation. In contrast, C/EBPε is up-regulated during differentiation. In order to determine whether the C/EBP transcription factors play a role in eosinophil differentiation of human $CD34^+$ cells, C/EBP proteins were inhibited during this process. For this purpose, we transduced $CD34^+$ cells with a dominant negative C/EBP protein (DN-C/EBP), which prevents DNA binding of endogenous C/EBP proteinss. Our experiments demonstrate that C/EBPs play a role in the proliferation and maturation during eosinophil differentiation. Ectopic expression of DN-C/EBP results in an inhibition of proliferation. Inhibition of C/EBP proteins results in a block of differentiation. These data clearly demonstrate that C/EBP transcription factors play a critical role in the differentiation of human eosinophils.

Materials & Methods

Cell isolation and Culturing

Isolation and culturing of human CD34 positive cells. CD34 positive cells were isolated as previously described [42]. In short, mononuclear cells were isolated from umbilical cord blood by density centrifugation over a ficoll-paque solution (density 1.077 g/ml). CD34 positive cells were isolated by MACS immuno-magnetic cell separation (Miltenyi Biotech, Auburn, USA) using a hapten conjugated antibody against CD34, which was coupled to magnetic beads. CD34 positive cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Paisly, UK) supplemented with 10% heat inactivated fetal calf serum (FCS; Life Technologies, Breda, The Netherlands), 50 mM b-Mercaptoethanol, 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies) and 2mM Glutamine (Gibco, Paisly, UK) at a density of 250.000 cells/ml. Cells were differentiated towards eosinophils upon addition of SCF (50 ng/ml Pepro Tech, London, UK), FLT-3 ligand (50 ng/ml Pepro Tech, London, UK), GM-CSF (0,1 nmol/l Endogen, Hmapshire, UK), IL-3 (0,1 nmol/l Strathmann, Hamburg, Germany), and IL-5 (0,1 nmol/l a kind gift from Dr. M. McKinnon, GSK, UK). Every 3 or 4 days, cells were counted and diluted in fresh medium to a density of 500.000 cells/ml and the cytokines IL-3 and IL-5 were added to the cells.

Culturing of Phoenix-A. The Phoenix-Ampho cells, a 293T-based amphotropic retroviral packaging cell line [43], were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 8% heat inactivated FCS (Life Technologies), 100 U/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies).

Constructs

We used a bicistronic retroviral DNA construct with a gene of interest and a marker gene encoding for eGFP (LZRS-IRES-eGFP) or murine CD8α (LZRS-IRES-Lyt-2) down-stream of an Internal Ribosomal Entry Site (IRES), allowing independent translation of the products of both genes in the transduced target cells. DN-C/EBP coding sequence was cloned from the pLNCX DN-C/EBP plasmid [44] in the *Nae I* site from the LZRS-IRES-eGFP to obtain the retroviral vector LZRS-DN-C/EBP-IRES-eGFP and from LZRS-DN-C/EBP-IRES-eGFP between the *BstB I* and *Not I* sites into the LZRS- IRES-Lyt-2 to obtain the retroviral vector LZRS-DN-C/EBP-IRES-Lyt-2.

The EDN promoter/intron (-300 to +298, GENBANK X16546) was cloned by PCR using human genomic DNA [45]. The amplified fragment was then sequenced and cloned into the promoterless luciferase reporter plasmid pGL3 (Promega).

Retroviral Transduction of CD34$^+$ cells

Helper-free recombinant retrovirus was produced by stable transfection of the amphotropic retroviral packaging cell line, Phoenix-ampho, by calcium phosphate co-precipitation. Cells were plated in 6 cm dishes 24 hours before transfection. 5 Minutes prior to transfection 25 mM Chloroquine diphosphate was added to the cells. A total of 10 μg of DNA was used per transfection. Medium was refreshed, 16 hours after transfection. After an additional 24 hours, cells were split into 75 cm^2 dishes (Greiner, Alphen a/d Rijn, The Netherlands) and 2 mM Puromycin was added to the cells. After two weeks of selection, cells were plated into 25 cm^2 dishes (Greiner) and were grown to a confluence of 90%. Subsequently cells were grown in a minimal amount of medium for 24 hours. Viral supernatants were collected and filtered through a 0.45 μm filter.

CD34 positive cells were transduced after two days of differentiation in 24 wells dishes coated with 20 μg/ cm^2 recombinant human fibronectin fragment CH-296 (RetroNectin; Takara, Otsu, Japan), and 2% Bovine Serum Albumin (BSA). Transduction was performed by addition of 0.5 ml viral supernatant to 0.5 ml medium containing 500.000 cells. 24 Hours after transduction 0.7 ml medium was removed from the cells and 0.5 fresh virus supernatant was added to the cells together with 0.5 ml fresh medium and cytokines. The percentage of eGFP positive, living cells was determined every 3 or 4 days by FACS-analysis (FACSvantage, Becton Dickinson, San Jose, CA) containing 1 μg of propidium iodide (PI)/ml.

Immuno-histochemistry staining of cytospins

The percentage of eosinophil differentiation was determined by histochemical staining of the cells. Cytospins were prepared from 5 x 10^4 differentiated eosinophils. Slides were dried on silica gel for 24 hours before fixing them in dry-acetone for 10 minutes. The percentage of cells differentiating towards eosinophils was determined by Luxol Fast Blue staining (Avocado Res. Chem. Ltd., Heysham, UK), a dye specifically staining granules of eosinophils. Slides were stained with 0.15 % w/v Luxol Fast Blue in urea saturated ethanol for 2 hours.

Giemsa May-Grünwald staining was also used to analyze differentiating eosinophils. Cytospins were stained in a 50% Eosin Methylene Blue solution according to May-Grünwald (Sigma-Aldrich GmbH, Seelze, Germany) for 20 minutes, rinsed in water for 5 seconds, and the nuclei were counter-stained with 10% Giemsa solution (Merck kGaA, Darmstadt, Germany) for 15 minutes. During eosinophil differentiation, different stages of maturation can be observed. Cells differentiate from blast cells towards pro-myelocyte type I, pro-myelocyte type II, myelocyte, meta-myelocyte, and finally mature eosinophils with segmented nuclei. These stages can be distinguished by the size of the cells, ratio of cytoplasm versus nucleus present, presence of azurophilic granules, appearance of eosinophilic granules and the shape of the nuclei. ‘Juvenile’ eosinophils were, in our experiments characterized as cells belonging to the stages of pro-myelocyte II, myelocyte, metamyelocyte and mature eosinophils. These cells were all observed to contain eosinophilic granules.

Transient transfection and Luciferase assay

Transfection of in vitro differentiated eosinophils was performed by electroporation at 300 V and 950 μF (5.10^6 cells, 10 μg supercoiled plasmid DNA) [44]. 24 hours after transfection, cells were harvested and lysed in commercially available luciferase lysis buffer. CMV-Renilla co-transfection was used to correct for variations in transfection efficiency. Luciferase activity was measured with the Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany).

Western Blotting

For the detection of C/EBPα, C/EBPε, and DN-C/EBP differentiating eosinophils were lysed in Laemmli sample buffer (0.12 M Tris/HCl [pH 6.8], 4% SDS, 20% glycerol, 0.05% bromophenol blue, and 35μM β-mercaptoethanol). Total cell lysates were boiled for 5 min at 95° C and equal amount of protein were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (10mM Tris/HCl [pH 8.0], 150 mM NaCl and 0.3% Tween-20) supplemented with 5% dried non-fat milk. Blots were incubated overnight at 4° C with appropriate antibodies (1:1000) and after hybridization with secondary antibodies developed utilizing Enhanced Chemiluminescence (ECL, Amersham. UK).
The following antibodies were used: anti-C/EBPα (rabbit polyclonal IgG 14aa, sc-061X; Santa Cruz, San Jose,CA), anti-C/EBPε (rabbit polyclonal IgG C-22, sc-158X; Santa Cruz).

Apoptosis

For the analysis of apoptosis by Annexin-V staining cells were washed with ice-cold PBS, resuspended in binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, and 2.5 mM $CaCl_2$). Cells were then incubated with phycoerythrin (PE)-conjugated Annexin-V for 30 min on ice and fluorescence was analyzed by FACS-analysis (FACSvantage, Becton Dickinson, San Jose, CA), by measuring a total cell count of 10,000 cells per sample.
For detection of Lyt-2, cells were stained with fluorescein isothiocyanate (FITC)-conjugated Lyt-2 antibody (BD Pharmingen). After staining, cells were washed twice in phosphate-buffered saline (PBS) and analyzed by flow cytometry. All steps of the staining procedure were performed on ice, and appropriate fluorochrome-conjugated, isotype-matched control antibodies were used in all experiments.

Reverse transcriptase-PCR (RT-PCR) assays.

RNA was isolated from FACS-sorted differentiating eosinophils as described previously [46], reverse transcribed using a poly-dT_{15} oligonucleotide (Invitrogen, Breda, The Netherlands) and 400 U of Maloney murine leukemia virus (M-MuLV) reverse transcriptase (Invitrogen) at 37° C for 1 hour. Semi-quantitative PCR assays were performed in 30 mL reaction volumes using the appropriate diluted amounts of cDNA template, 2 mmol/L $MgCl_2$, 0.25 mmol/L each dNTP, 10

pmol/L of each primer, and 0.8 U Taq polymerase (Roche, Basel, Switzerland) in 1x buffer (10 mmol/L Tris-HCl, pH 8.5, 50 mmol/L KCl). Reaction conditions were a 10 minute denaturing step at 94° C followed by 30 cycles of 1 minute at 95° C, 1.5 minuteat 56° C, and 2 minute at 72° C. PCR products were separated on1% agarose gels, stained with ethidium bromide. Three separate FL donor pools were analyzed and each PCR was repeated 4 to 6 times. The βc, IL-5Rα and β-actin primers that were used are as follows: βcR: CTGTGGCTATACCCATGAGC; βcR: TCAAACCCTGACTTCACAGG; IL-5RαF: CACACTGGGGAGAAATATCG; IL-5RαR: CGAGTTCCTTCAATCTCTGC. For β-actinF and β-actinR primers see [47].

Results

Expression pattern of C/EBPα and C/EBPε during eosinophil differentiation of CD34⁺ cells.

Members of the C/EBP transcription factor family play a role in the differentiation and lineage commitment of eosinophils. In mice and human cell lines C/EBPα is expressed in early myeloid progenitors, and its expression decreases as these progenitors differentiate into mature granulocytes [48]. In turn, C/EBPε is up-regulated at the promyelocyte or myelocyte stage, as shown in mice and human cell lines [28; 49]. To investigate the role of C/EBP transcription factors in human eosinophil development derived from CD34⁺ cells, we first examined the expression levels of C/EBPα and C/EBPε. Therefore, CD34⁺ progenitor cells isolated from umbilical cord blood were differentiated towards eosinophils in the presence of the cytokines IL-3 and IL-5. Protein lysates were made at different time-points during differentiation. Proteins were separated on 12% SDS-PAGE by electrophoresis and western blotting was performed with antibodies against C/EBPα and C/EBPε. As shown in figure 1A C/EBPα is expressed up to day 7 and expression decreases during differentiation, but remains present. C/EBPε levels are increasing from day 3 to 14 and remains expressed at a high level (Fig. 1B). These experiments show that in this human *ex vivo* system C/EBPα is expressed at high levels upon induction of differentiation and is down-regulated with maturation. On the other hand, C/EBPε is induced during differentiation of CD34⁺ cells *in vitro* and remains at a high level. This is in agreement with the data obtained from aforementioned cell lines

Retroviral transduction of CD34⁺ cells with DN-C/EBP.

In order to investigate the role of C/EBP proteins during differentiation of CD34⁺ cells towards eosinophils we used a C/EBP construct which act as a dominant negative variant, because the basic region has been replaced with a designed acidic amphipathic helix [50]. This alteration prevents the basic region from binding to DNA and therefore inhibits DNA binding of endogenous

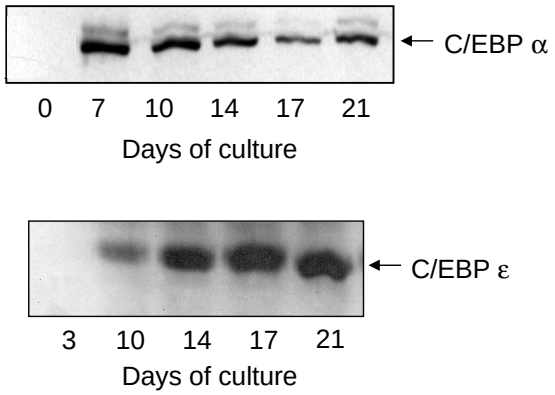

Figure 1 Altered expression of C/EBPα and ε in differentiating eosinophils. CD34+ cells isolated from cord blood were differentiated towards eosinophils. Protein lysates were made at day 7, 10, 14, and 17. Western Blot analysis was performed with an antibody against C/EBPα (Fig 1A, top panel) and an antibody against C/EBPε (Fig. 1B, bottom panel). The experiment shown is representative of three additional experiments.

C/EBPs. To show the effect of this construct on transcription activation, isolated CD34+ cells were differentiated towards eosinophils and after 14 days transfected with the construct containing DN-C/EBP and a reporter construct. The luciferase reporter construct is down-stream of the EDN promoter, which is under the control of C/EBP transcription factors [44]. Two days after transfection luciferase activity was measured. Figure 2A shows an inhibition of EDN promoter activity of approximately 80%, the amount of inhibition is comparable to the eosinophilic HL-60-eos cell line, which we have shown before [44]. We cloned the dominant negative C/EBP into a retroviral vector, this construct contains beside the DN-C/EBP construct also a marker, eGFP (Fig. 2B; upper scheme) or the extracellular part of murine CD8 (Fig. 2B; lower scheme). The marker is downstream of an Internal Ribosomal Entry Site (IRES), allowing independent translation of the products of both genes. We differentiate isolated CD34+ cells towards eosinophils with IL-3 and IL-5 and transduced them at day two with the viral construct containing DN-C/EBP. After three days the percentage of eGFP positive cells was determined by FACS analysis, transduction efficiencies were 45.9% ± 1.5% (n = 8; a representative experiment is shown in Fig 2C). Expression of DN-C/EBP in transduced cells was confirmed by western blotting of protein lysates from transduced cells with a C/EBPα antibody, which is cross-reactive with DN-C/EBP (fig. 2D). Taken together, we showed that a DN-C/EBP construct inhibits the transactivation of EDN, which is under the control of C/EBP proteins, in CD34+ cells differentiated with IL-3 and IL-5. CD34+ cells express DN-C/EBP after viral transduction.

Inhibition of C/EBP proteins in differentiating eosinophils results in inhibited proliferation, but not cell death.

To determine the role of C/EBP transcription factors in regulating eosinophil differentiation, isolated CD34$^+$ cells were transduced with virus obtained from cells transfected with either LZRS-IRES-eGFP or LZRS-DN-C/EBP-IRES-eGFP. Proliferation was determined by counting the total amount of eGFP positive cells. Transduction of cells with the empty vector resulted in a proliferation of approximately 30 fold between day 7 and day 21. In contrast, transduction of cells with DN-C/EBP resulted in a diminished proliferation between day 7 and day 21 of approximately

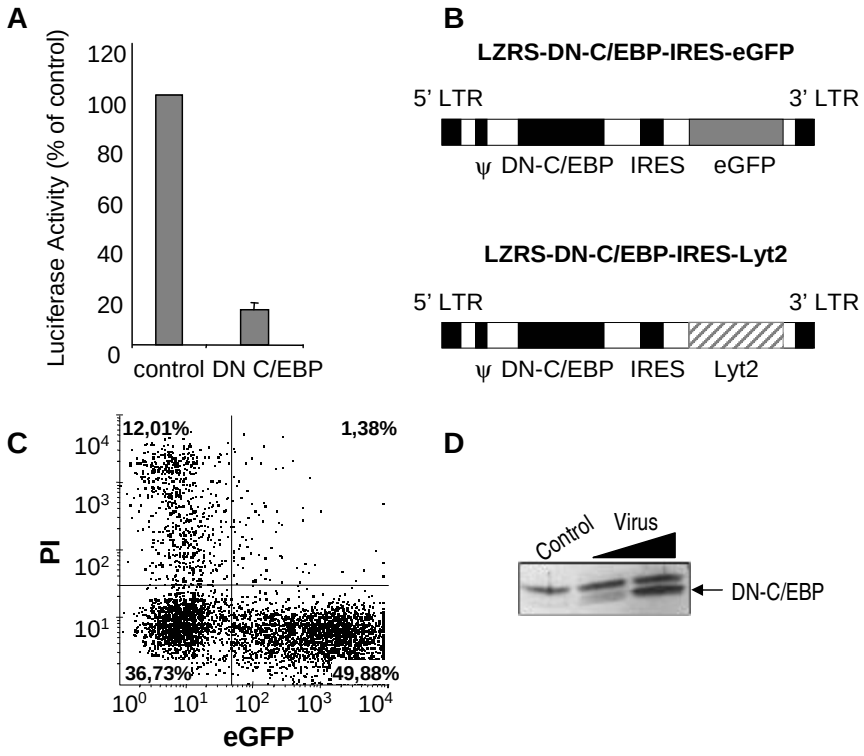

Figure 2 Retroviral transduction of CD34$^+$ cells with DN-C/EBP. A CD34+ cells were differentiated towards eosinophils and transfected at day 14 with DN-C/EBP or the empty vector. Two days after transfection luciferase activity was measured. Results are expressed as percentage ± S.E.M. of four individual experiments. **B** A schematic representation of the LZRS DN-C/EBP constructs. The DN-C/EBP has been designed by replacing the basic region of C/EBP α with an acidic amphipatic helix. Therefore it can bind to all B-ZIP transcription factors and prevents DNA binding. As marker genes eGFP (top panel) or Lyt-2 (bottom panel) was used, which were under the control of an Internal Ribosomal Entry Site (IRES). **C** CD34+ cells were transduced with retrovirus and three days after transfection measured on the FACS, a representative figure is shown. **D** CD34+ cells were transduced with virus from QNX-A cells transfected with the LZRS-IRES-eGFP vector (1) or with the LZRS-DN-C/EBP-IRES-eGFP (2.10^5 (2) or 1.10^6 (3) infectious virus particles/ml). Three days after transduction cells were lysed and samples were analysed by SDS-PAGE. The experiment shown is representative of three additional experiments.

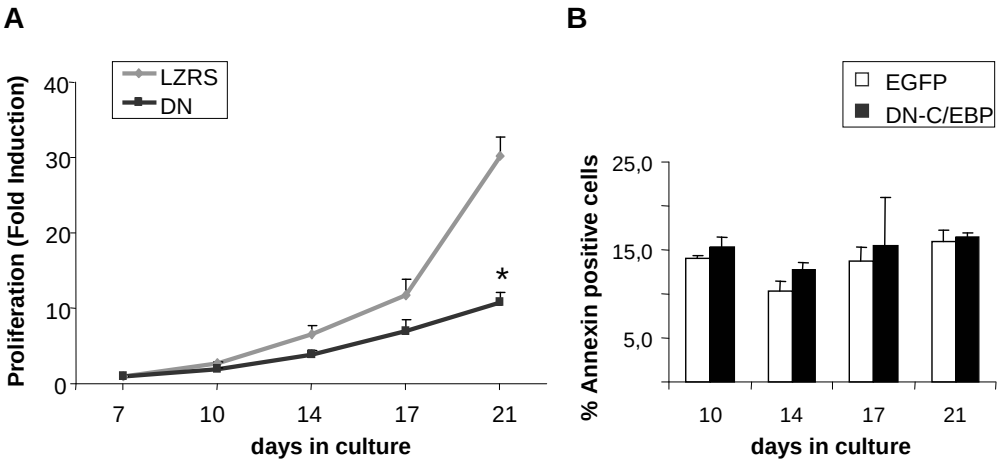

Figure 3 Ectopic expression of DN-C/EBP results in inhibition of proliferation. A CD34+ cells were transduced with LZRS-IRES-eGFP or LZRS-DN-C/EBP-IRES-eGFP. Proliferation was determined by counting the eGFP positive cells. Results are expressed as fold increase in cell numbers compared with day 7 as an average ± S.E.M. of five individual experiments. B CD34+ cells were transduced with LZRS-IRES-Lyt-2 or LZRS-DN-C/EBP-IRES-Lyt-2. At day 10, 14, 17, and 21 cells were stained with an FITC-labeled antibody against Lyt-2 and a PE-labeled antibody against Annexin-V. Results are expressed as an average ± S.E.M. of three individual experiments. * Indicates a significant difference compared with the EGFP control $P < 0.05$.

10 fold (figure 3A). These data demonstrates that C/EBPs are involved in the regulation of proliferation during eosinophil differentiation.

To investigate if the decrease in proliferation of cells transduced with DN-C/EBP was due to a stop in proliferation or enhanced cell death we measured the amount of annexin-V positive cells during the differentiation of transduced cells as a read-out of early apoptosis. Surprisingly, we could not detect apoptosis in cells expressing eGFP. As shown in fig 2C hardly any cell was double positive for eGFP and PI. Also detection of annexin-PE and eGFP appeared not to be possible. Together these data indicate that soluble eGFP leaks from dying cells. This was also mentioned by Harvey *et al*. [51].Therefore, we transduced cells with the virus obtained from cells transfected with LZRS-IRES-Lyt-2 or LZRS-DN-C/EBP-IRES-Lyt-2, because Lyt-2 is membrane-bound and therefore can not leak out of the cell.

As shown in figure 3B there was no significant difference in the amount of annexin-V positive cells between the LZRS-IRES-Lyt-2 transduced cells and the cells transduced with LZRS-DN-C/EBP-IRES-Lyt-2. These results indicate that the decrease in the proliferation of cells transduced with DN-C/EBP was not mediated by accelerated apoptosis.

Transduction of DN-C/EBP avoids eosinophilic differentiation of CD34⁺ cells.

In order to investigate if C/EBP proteins play a role in eosinophil differentiation, CD34⁺ cells were differentiated with IL-3 and IL-5 towards eosinophils and transduced with either LZRS-IRES-eGFP or LZRS-DN-C/EBP-IRES-eGFP virus. After 10, 14, 17, and 21 days of differentiation, eGFP positive cells were sorted by the FACS from the non-transduced cells and cytospins were prepared. The morphology of the differentiating eosinophils was analyzed by histological May-Grünwald Giemsa staining. Differentiation of cells transduced with eGFP resulted in about twenty percent juvenile eosinophils at day 10 and increases up to nearly 75% after 21 days (fig. 4A). On the other hand transduction of cells with DN-C/EBP resulted, after 21 days of differentiation, in a low percentage of juvenile eosinophils of approximately four percent. The cells remain in the blast stage.

To further characterize the role of endogenous C/EBP in eosinophil differentiation, cytospins of transduced cells were also analyzed by Luxol Fast Blue (LFB) staining, an eosinophil granule-specific dye. After 10 days of differentiation, about 33% of the cells transduced with eGFP were positive for LFB. This percentage increased to almost 70% after 21 days of differentiation. In contrast, differentiation of cells transduced with DN-C/EBP results in approximately 10% LFB positive cells after 21 days (fig. 4B). These data indicate that C/EBP proteins play an important role in eosinophil differentiation.

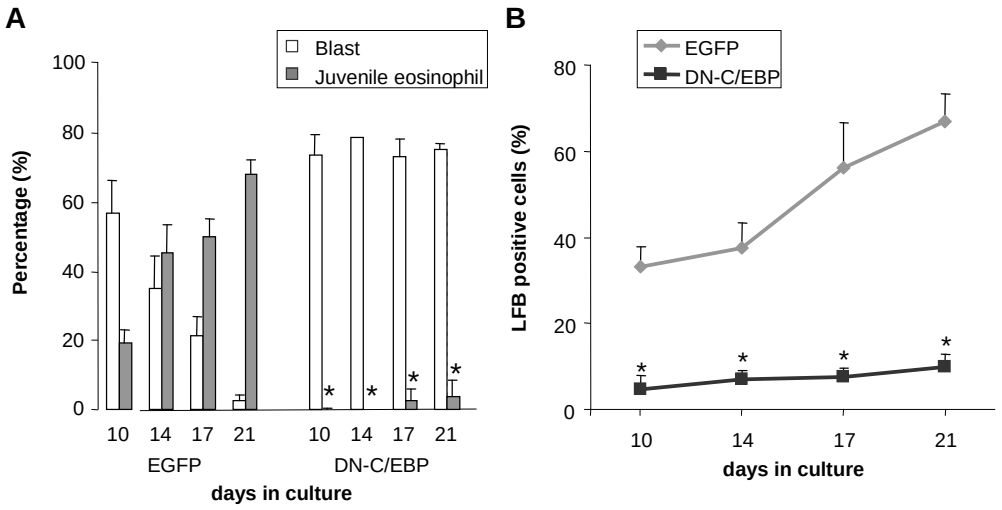

Figure 4 Ectopic expression of DN-C/EBP results in inhibition of differentiation. CD34⁺ cells were transduced with LZRS-IRES-eGFP or LZRS-DN-C/EBP-IRES-eGFP. After 10, 14, 17, and 21 days of differentiation transduced cells were separated from the non-transduced cells, and cytospins were made. **A** Cytospins were stained with the Giemsa May-Grünwald solution. The percentage of progenitors and juvenile eosinophils was expressed as an average ± S.E.M. of five individual experiments. **B** Cytospins were stained with the Luxol Fast Blue for 2 hours after fixation with dry-acetone and determined by microscopy. The percentage of LFB positive cells was expressed as an average ± S.E.M. of four individual experiments. * Indicates a significant difference compared with the EGFP control $P < 0.05$.

Reduced expression of IL-5R on CD34+ cells differentiated towards eosinophils in the presence of DN-C/EBP.

C/EBPα knockout mice have greatly reduced granulocyte colony stimulating factor receptor (G-CSFR) expression and responsiveness [38]. Stimulation of CD34+ cells with G-CSF results in neutrophils [21]. The IL-5R consist of a common β chain which is shared with the receptors for IL-3 and GM-CSF and a specific α chain [52; 53]. Since we have shown before that C/EBP is involved in the regulation of transcription of the common beta chain, we want to examine the role of C/EBP proteins on expression of the IL-5 receptor (IL-5R) [54]. For this purpose, we transduced CD34+ cells cultured with IL-3 and IL-5 with either eGFP or DN-C/EBP and after 10 days eGFP positive cells were sorted by the FACS from the non-transduced cells. RNA was isolated from these cells and a semi-quantitative RT-PCR was performed. As shown in figure 5 cells transduced with DN-C/EBP show a marked decrease in IL-5β mRNA expression. We also found a reduction of the amount of IL-5Rα mRNA. These results indicate that DN-C/EBP inhibits the expression of mRNA of both the IL-5Rα and the IL-5Rβ chain in transduced cells.

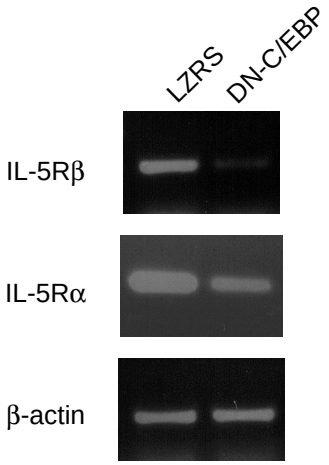

Figure 5 Inhibition of C/EBP proteins results in down-regulation of βc mRNA. CD34+ cells were transduced with LZRS-IRES-eGFP or LZRS-DN-C/EBP-IRES-eGFP. After 10, 14, 17, and 21 days of differentiation transduced cells were separated from the non-transduced cells, RNA was isolated and semi-quantitative PCR assays were performed using gene-specific primers. Each of the RT-PCR reactions was performed with two different batches of RNA.

Discussion

In this report we have investigated the role of the C/EBP transcription factors during eosinophil differentiation. We showed the expression patterns of C/EBPα and C/EBPε during eosinophil differentiation. We have shown that the expression of C/EBPα is high during the early stages of eosinophil differentiation By contrast, C/EBPε seems more involved in the latter maturation of eosinophils because of its augmented expression during differentiation. These patterns were illustrated before; however these experiments were done in mice and in cell lines [28; 48; 49].

In order to examine the effect of inhibition of C/EBP, we used an *ex-vivo* differentiation system of umbilical cord blood cells. CD34+ cells were isolated and differentiated towards eosinophil in

the presence of IL-3 and IL-5, which results in a high percentage of juvenile eosinophils [55]. The multiplicity of the C/EBP family made it necessary to make use of a dominant inhibitory protein for this purpose, which prevents DNA binding of endogenous C/EBPs. The presence of several C/EBPs in the myeloid lineages suggests that related family members might compensate in vivo for the lack of a single isoform, just as GATA family members partially compensate for the lack of GATA-1 [56]. Potential redundancy is evident from the ability of C/EBPβ to compensate for the loss of C/EBPα in hepatocytes and from the ability of both C/EBPα and C/EBPε to direct the granulocytic differentiation of myeloblastic cell lines [57-60]. The ability of DN-C/EBP to interfere with the multiple C/EBPs avoids compensatory effects that can arise in gene knockouts.

Retroviral transduction experiments were performed in human $CD34^+$ cells to express a dominant negative C/EBP. Specificity of DN-C/EBP was demonstrated in differentiating eosinophils by inhibition of transcription activity of the EDN gene (see fig 2A). We examined the effect of C/EBP proteins on eosinophil differentiation. The differentiation judged by the morphology of the cells was strongly inhibited in cell transduced with the retrovirus containing DN-C/EBP. This is consistent with the findings in knock-out mice and the involvement of C/EBP proteins in the regulation of lineage-specific proteins [38]. C/EBPα knockout mice lack neutrophils and eosinophils but retain monocytes, lymhocytes, erythroid cells and immature myeloblasts [38]. They do not develop granulocytic colony forming units *in vitro* [38; 61]. C/EBP ε knockout mice are normal at birth and fertile, however, they fail to produce eosinophils. Neutrophils are present in deficient mice, but have an atypical nuclear morphology compared with wild-type neutrophils [62]. C/EBP transcription factors are also involved in the activation of genes specific in the eosinophilic lineage, like EDN, MBP and eos47(avian) [40; 44; 63]. Taken together, C/EBP proteins are important regulators of eosinophilic differentiation, as inhibition results in a block at the stage of early progenitors.

Transduction of differentiating eosinophils with DN-C/EBP, results in an inhibition of both proliferation and differentiation. This is a remarkable result, because generally induction of differentiation is accompanied by a decrease of proliferation. The inhibition of proliferation and differentiation might be due to the design of the DN-C/EBP which is based on C/EBPα. It is known that C/EBPα is an inhibitor of cell proliferation when over-expressed in cultured cells [30; 64]. In addition, hepatocytes from newborn C/EBPα-deficient mice display increased proliferative activity [65]. Although C/EBPα is a transcription factor, its ability to arrest growth does not require its DNA binding activity, but is mediated via protein-protein interaction [66]. C/EBPα has been found to interact with several proteins that are involved in the control of cell cycle progression, thereby indicating the existence of multiple pathways through which C/EBPα mediates growth arrest [66-71]. C/EBPα may inhibit the proliferation via interaction with E2F or with cdk2 [68; 72-74]. However, interaction with cdk2 occurs via the C/EBPα basic region, and this segment is altered in DN-C/EBP [74]. Taken together this might indicate that the DN-C/EBP, which dominant negative is for transcriptional activity of C/EBP family members, acts as an activator for protein-protein

interactions. However, we can not rule out that the C/EBP proteins are involved in the proliferation by gene regulation.

Inhibition of C/EBP activity during eosinophil differentiation results in strong inhibition of the expression of endogenous IL-5Rβ RNA. This is consistent with the finding that C/EBPα directly activates the IL-5Rβ promoter [54]. IL-5$^{-/-}$, IL-5Rα$^{-/-}$, and $\beta_c^{-/-}$ mice retain lower, but detectable levels of eosinophil [75-77]. In addition, in neutrophils C/EBPα is involved in activation of the promoter of the granulocyte colony stimulating factor receptor (G-CSFR) [34; 35]. G-CSF is a lineage-specific cytokine involved in the differentiation of neutrophils. C/EBPα knockout mice lack G-CSF responsive progenitors and have no mature neutrophils and no mature eosinophils [38]. This might indicate that C/EBP proteins play a role in the differentiation of both neutrophils and eosinophils by regulation of the receptor involved in specific differentiation signals. IL-5 has been shown to regulate the expression of the IL-5 receptor α-subunit [78]. We also observed a decrease in the expression of IL-5Rα mRNA. This might be due to the reduced transcription of the βc gene and impaired IL-5 signaling.

In summary, differentiation of human primary eosinophils is controlled by C/EBP transcription factors. Proliferation of human CD34+ cells was severely inhibited by the expression of a DN-C/EBP. Cells expressing DN-C/EBP remain in the blast stage, did not undergo apoptosis. Furthermore, we conclude that C/EBPs contribute to eosinophilopoiesis via induction of the common β gene. Future experiments will focus on identifying the role that each C/EBP isoform plays in these regulatory processes.

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Chapter 6

C/EBP epsilon is rate-limiting in G-CSF induced Granulocyte Differentiation

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In preparation

Abstract

Granulocyte colony-stimulating factor (G-CSF) is the major regulator of granulopoiesis and acts through binding to its specific receptor (G-CSF-R) on neutrophilic progenitors. G-CSF induces granulocytic differentiation in the murine myeloid cell line 32D. Hematopoietic differentiation is regulated by lineage-specific transcription factors, such as C/EBPε. We have investigated the role of the C/EBPε transcription factor in the G-CSF induced granulocytic differentiation of the murine 32D cell line. To investigate the role of C/EBPε in the early differentiation of granulocytes, we used a 4-hydroxy-tamoxifen inducible system allowing the activation of C/EBPε within minutes. Cells expressing C/EBPε cultured in the presence of G-CSF showed an accelerated differentiation after 24h, both on granulocyte-specific marker expression and morphology. Using the system of 4-hydroxy-tamoxifen induced C/EBPε activation we showed that C/EBPε is rate-limiting in G-CSF induced granulocytic differentiation. Furthermore, we show a decreased proliferation of 32D cells expressing C/EBPε cultured in the presence of G-CSF. These results demonstrate that C/EBPε and G-CSF cooperate in the induction of granulocytic differentiation. Expression of C/EBPε turns out to be rate-limiting in the differentiation of granulocytes.

Introduction

The maintenance of adequate numbers of terminally differentiated hematopoietic cells during health and or disease is ultimately regulated by the continuous self-renewal, controlled proliferation, and differentiation of hematopoietic stem cells [1; 2]. Closely regulated expression of cytokines, colony-stimulating factors, receptors, and transcription factors coordinate lineage differentiation from pluripotent stem cells into erythroid, myeloid, and lymphoid precursors [3; 4].

In the myeloid lineage CCAAT/enhancer-binding proteins (C/EBP) play a role in the differentiation. The C/EBPfamily consists of six members, including the highly related C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε, and the less related C/EBPγ and C/EBPζ [5-12]. They belong to the basic region/leucine zipper (bZIP) family of transcription factors. C/EBP proteins contain a basic amino acid domain and a leucine zipper region allowing the formation of homo- and heterodimers [13]. They share similar DNA binding specificities (13). The C/EBPs serve as critical regulators in the differentiation processes of a variety of mammalian cells including adipocytes, hepatocytes, and myeloid cells [14; 15]. In contrast to the other C/EBP members, which are widely expressed, C/EBPε is only expressed at high levels in the late stages of granulopoiesis and in T-lymphocytes [16].

The C/EBP family members are known to exert pleiotropic effects in the tissues in which they are expressed [17]. This effect may be due to their tissue- and differentiation-stage specific expression, their ability to dimerise with members of their own family of transcription factors, and

their capability to interact with other transcription factors [13; 17]. C/EBPε is essential for terminal differentiation and functional maturation of committed granulocyte progenitor cells (18). C/EBPε has been identified as a critical regulator of terminal granulopoiesis and one of the causative mutations in the human disease, neutrophil-specific granule deficiency [19-21].

Granulocyte-Colony Stimulating Factor (G-CSF) stimulates both the proliferation and differentiation of granulocyte precursor cells [22-24]. It also stimulates a variety of responses in mature neutrophils, including prolonged survival, phagocytosis, and superoxide production [25-27]. G-CSF binds to its receptor (G-CSFR) on the cell surface to form a homodimeric receptor complex [28]. The membrane-proximal region of G-CSFR has been shown to be essential for the transmission of growth signals via JAK2 activation, STAT3 and STAT5 phosphorylation, and MAP kinase phosphorylation [29-32]. Accumulated evidence indicates that G-CSF and its receptor are essential for hematopoietic development and that G-CSF may be implicated in malignant disease [29; 33].

The precise role that cytokines play in the early commitment to a particular cell lineage remains to be fully defined. It is still an open issue whether cytokines are involved in the lineage decisions of multipotent cells by directing a particular cellular pathway or on the other hand merely support the survival of cells that have intrinsically selected a specific hematopoietic lineage [34; 35].

In the present study we have investigated the role of C/EBPε in the differentiation of 32D cells. We especially focused on the effects of C/EBPε on the induction of differentiation. Therefore we used a tamoxifen-inducible system, which is characterized by a silenced transcriptional activity of C/EBPε until tamoxifen is added. We show that both G-CSF signaling and C/EBPε are important to induce granulocytic differentiation in 32D cells and C/EBPε is rate limiting in this process.

Materials & Methods

Cell Culturing

32D.C8.6, a subline of the IL-3-dependent murine myeloid cell line 32D, stably expressing the human G-CSF-R, was generated as described previously [36]. Cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS;Gibco), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), 2mM Glutamine (Gibco) and recombinant mouse IL-3 produced in COS cells [37].

Facs analysis and proliferation assay

To induce differentiation, the cells were washed twice with phosphate-buffered saline and cells were incubated at an initial density of 1×10^5 cells/mL in 10% FBS/RPMI medium supplemented with murine IL-3, 100ng/mL of human G-CSF (a kind gift of Dr R. Fijnheer, UMC) or 100ng/mL of human G-CSF and 500 nM 4-Hydroxy-tamoxifen (4OHT; Sigma) The medium was replenished every 2 to 4 days, and the cell densities were adjusted to 1 to 2×10^5 cells/mL. Viable cells were counted every 24 h by trypan blue exclusion.

For the analysis of GR-1 expression a terminal differentiation marker, differentiating 32D C/EBPε-ER cells were washed with ice-cold PBS, stained with R-phycoerythrin (PE)-conjugated Ly-6G (Gr-1; BD Pharmingen) After staining, cells were washed twice in phosphate-buffered saline (PBS) and antibody fluorescence was analyzed by FACS-analysis (FACSvantage, Becton Dickinson), by measuring a total cell count of 10,000 cells per sample.

Constructs and Generation of stable cell lines

pSG5 C/EBPε-ER was generated by cloning C/EBPε without the stop codon into SK^+-ER [67]. The C/EBPε-ER was cloned by PCR into the pSG513 vector and verified by sequencing. To obtain 32D cells stably expressing C/EBPε-ER 5×10^6 32D cells were electroporated (280 V, 950 μF) with 10 μg expression vector and selected in 500 μg/mL G418 (Boehringer GmbH) in the presence of murine IL-3. The clones were obtained by limiting dilution and expression of C/EBPε-ER was examined using Western blot analysis (see below).

Immuno-histochemistry staining of cytospins

The percentage neutrophil differentiation was determined by histochemical staining of the cells. Cytospins were prepared from 5×10^4 differentiated 32D cells. Slides were dried on silica gel for 24 hours before fixing them in dry-acetone for 10 minutes. Giemsa May-Grünwald staining was used to analyze 32D cells differentiating towards neutrophils. Cytospins were stained in a 50% Eosin Methylene Blue solution according to May-Grünwald (Sigma-Aldrich GmbH) for 20 minutes, rinsed in water for 5 seconds, and the nuclei were counter-stained with 10% Giemsa solution (Merck kGaA) for 15 minutes. During neutrophil differentiation, different stages of maturation can be observed. Cells differentiate from blast cells towards cells with a banded nucleus, a segmented nucleus and finally neutrophils with highly-segmented nuclei. These stages can be distinguished by the size of the cells, ratio of cytoplasm versus nucleus, neutrophilic granule-containing cytoplasm, and the shape of the nuclei.

Western Blotting

For the detection of C/EBPε-ER 32D cells were lysed in Laemmli sample buffer (0.12 M Tris/HCl [pH 6.8], 4% SDS, 20% glycerol, 0.05% bromophenol blue, and 35μM β-mercaptoethanol). Total cell lysates were boiled for 5 min at 95° C and equal amount of protein were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with blocking buffer (10mM Tris/HCl [pH 8.0], 150 mM NaCl and 0.3% Tween-20) supplemented with 5% dried non-fat milk. Blots were incubated overnight at 4° C with anti-ERα (MC-20, Santa Cruz) or anti C/EBPε (sc-158X, Santa Cruz) and after hybridization with secondary antibodies developed utilizing Enhanced Chemiluminescence (ECL, Amersham). Blots were subsequently probed with β-actin antibody (Santa-Cruz, sc-1616) to confirm equal protein loading.

Results and Discussion

C/EBPε is a downstream target of G-CSF signalling

G-CSF and C/EBPε play an important role in myeloid development. Mice deficient in either G-CSF or its receptor have a reduced amount of neutrophils (38-40). C/EBPε knockout mice have impaired granulopoiesis and myelodysplasia [41]. They produce morphologically and functionally abnormal granulocytes. In addition, functional loss of C/EBPε is causativein the development of neutrophil specific granule deficiency (SGD). SGD is a rare congenital disorder in which the neutrophils display atypical bilobed nuclei, lack expression of granule proteins; and possess defects in chemotaxis, disaggregation, receptor upregulation, and bactericidal activity. G-CSF induces granulocytic differentiation in the murine myeloid cell line 32D. Both G-CSF and C/EBPε

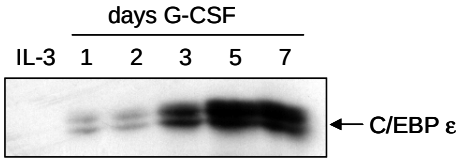

Figure 1. Upregulation of C/EBPε expression during granulocytic differentiation of 32D cells 32D cells were washed twice in PBS and cultured in the presence of G-CSF or IL-3. Cells were lysed at the indicated time-points and samples were analysed by SDS-PAGE. Western Blot analysis was performed with an antibody against C/EBPε. The experiment shown is representative of two additional experiments.

are regulators of myeloid differentiation in 32D cells. In medium containing IL-3, 32D cells grow and maintain blast-like morphologic characteristics. However when exposed to G-CSF the 32D cell line differentiated to neutrophils [33]. We used a 32D clone which lacks the murine G-CSF receptor, but it ectopically express the human G-CSF receptor. We first addressed the question whether G-CSF induces C/EBPε in 32D cells. Therefore, we cultured 32D cells in the presence of G-CSF to induce granulocytic differentiation. When cultured with IL-3 there is no expression of C/EBPε (figure 1). G-CSF clearly induced C/EBPε expression in 32D cells after one day of stimulation. Expression reached the maximum level in 5 days. These results indicate that C/EBPε is upregulated during G-CSF induced granulocytic-differentiation.

Expression of Inducible C/EBPε Construct in 32D Cells

To better define the role of C/EBPε in differentiation processes in granulocytes, we used an inducible system. This system uses the expression of a fusion between C/EBPε and a mutated ligand-binding domain from the estrogen receptor (ER). This protein is silenced by binding of heat-shock proteins and therefore not functional. In the absence of the inducer, 4-hydroxy-tamoxifen (4-OHT), the C/EBPε-ER fusion is bound to heat-shock proteins. Addition of 4-OHT results in release of the heat-shock proteins from the C/EBPε-ER fusion protein. Using this inducible-system gave us the opportunity to examine the direct effects of the expression of C/EBPε on granulocyte development. Generation of stable cell lines takes weeks. Therefore, the first effect of the protein can not be examined. In contrast, addition of 4-OHT to the inducible system results in the rapid release of C/EBPε and effects are detectable within minutes.

To investigate the role of C/EBPε in granulocytic differentiation, we stably expressed C/EBPε-ER or the ER segment alone in 32D cells. By limiting dilution we obtained 17 clones, which were assessed for expression of the introduced proteins by western blotting (Figure 2). We did not observe any difference in the proliferation of the 32D C/EBPε-ER compared to the parental 32D or 32D ER cells in IL-3 (data not shown, fig 5). We have generated 32D clones expressing C/EBPε-ER, in which the activity of C/EBPε is silenced giving the possibility of investigation the direct actions of C/EBPε.

Effect of Inducible C/EBPε Expression on G-CSF-Induced Granulocyte Differentiation of 32D Cells

We next examined the correlation between C/EBPε expression and granulocytic differentiation. The expression of Gr-1, a GPI-anchored protein, is directly correlated with granulocyte differentiation and maturation [42]. Expression of the granulocytic marker GR-1 in response to G-CSF and 4-OHT was evaluated in these cell lines by FACS-analysis. There was no expression of GR-1 in both the 32D ER and the 32D C/EBPε-ER cells cultured in IL-3 (figure 3). After 3 days the expression of Gr-1 increases in 32D ER and in 32D C/EBPε-ER cells cultured in the

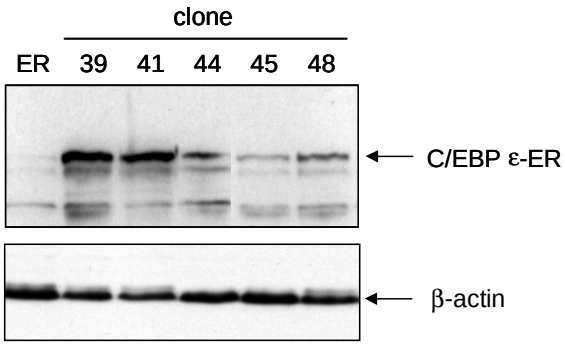

Figure 2. Generating stable cells expressing C/EBPε-ER. A 32D cells were transfected with C/EBPε-ER by electroporation. Clones were obtained by limiting dilution in the presence of G418. Selected clones were lysed and samples were analysed by SDS-PAGE. Western Blot analysis was performed with an antibody against the ER unit. The experiment shown is representative of two additional experiments. **B** Blot were subsequently probed with β-actin antibody to confirm equal protein loading.

presence of G-CSF (figure 3B and 3E). However in 32D C/EBPε-ER cells cultured in G-CSF in the presence of 4-OHT expression of Gr-1 was already enhanced after 24h (figure 3D). This enhanced expression of the differentiation marker Gr-1 was observed in all 17 different clones. In contrast in 32D ER cells cultured in the presence of G-CSF and 4-OHT there was no difference in the expression of Gr-1 compared to G-CSF alone (figure 3A). We show that induction of both G-CSF receptor signalling and C/EBPε resulted in an improved differentiation compared to differentiation induced with only G-CSF. This indicates that the induction of C/EBPε by G-CSF is an important step in the regulation of granulocyte differentiation. In response to different concentrations of 4-OHT 32D C/EBPε-ER cells showed dose-dependent differentiation in the presence of G-CSF (data not shown).

In contrast to our data, Nakajima and Ihle reported that expression of C/EBPε alone was sufficient to induce granulocytic differentiation in 32D cells [43]. In some clones we also observed an induced expression of the granulocytic marker Gr-1 after 7 days of culturing cells in the presence of 4-OHT. However, this induction was only observed in cells with a high expression level of C/EBPε-ER and not in cells with a lower expression level (figure 3C and 3F). This indicates that granulocyte differentiation induced by high levels of C/EBPε might be due to overexpression. In conclusion, we show that C/EBPε is rate-limiting in granulocytic differentiation of 32D cells.

Effect of Inducible C/EBPε Expression on Morphology of G-CSF-Induced 32D Cells

To further characterize the role of C/EBPε in the differentiation of neutrophils, we also examined the morphology of the cells during differentiation. Therefore we cultured 32D C/EBPε-ER and 32D-ER cells in G-CSF in the presence or absence of 4-OHT. We prepared cytospins at different time-points. The morphology of the differentiating 32D cells was analyzed by histological May-Grünwald Giemsa staining. Induction of differentiation of cells in the presence of G-CSF resulted in 21 percent banded neutrophils at day 1 and 20 percent segmented neutrophils after 3 days (fig. 4). Induction of differentiation of 32D C/EBPε-ER cells with G-CSF and 4-OHT resulted in 56 percent banded neutrophils at day 1 and 30 percent segmented neutrophils after 3 days. We show that beside the up-regulated expression of the granulocytic Gr-1 marker also morphologically the 32D C/EBPε-ER cells show an enhanced differentiation in the presence of G-CSF and 4-OHT.

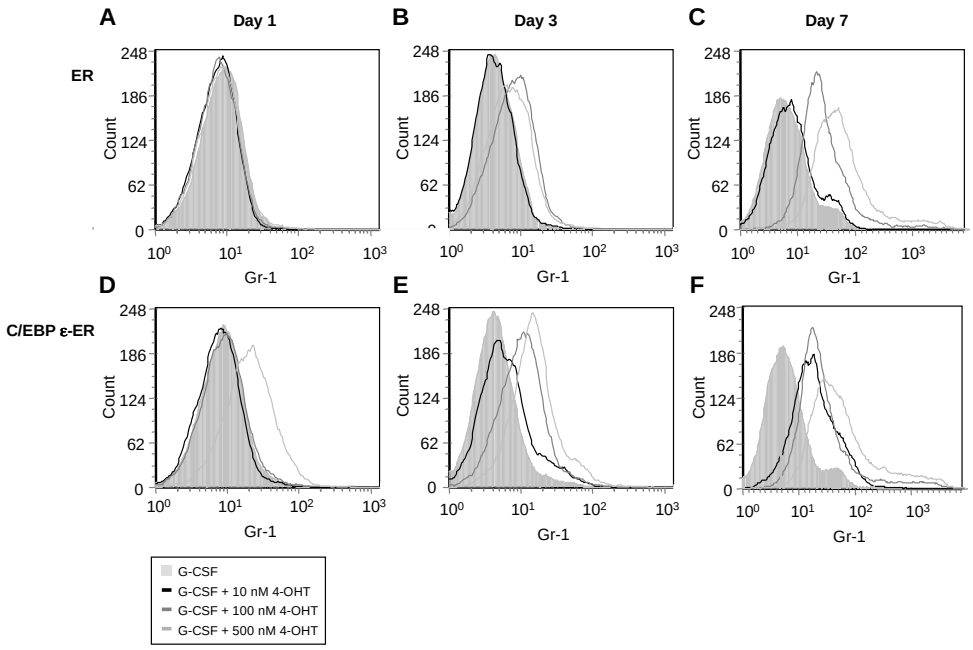

Figure 3. C/EBPε enhances G-CSF induced expression of granulocyte marker Gr-1. 32D-ER and 32D C/EBPε-ER cells were cultured in the presence of IL-3 or G-CSF in the presence or absence of 4-OHT. After 24h and 72 h cells were washed, stained with a PE-labelled antibody against Gr-1. All procedures were performed at 4° C. Cells were measured on the FACS and a representative experiment is shown.

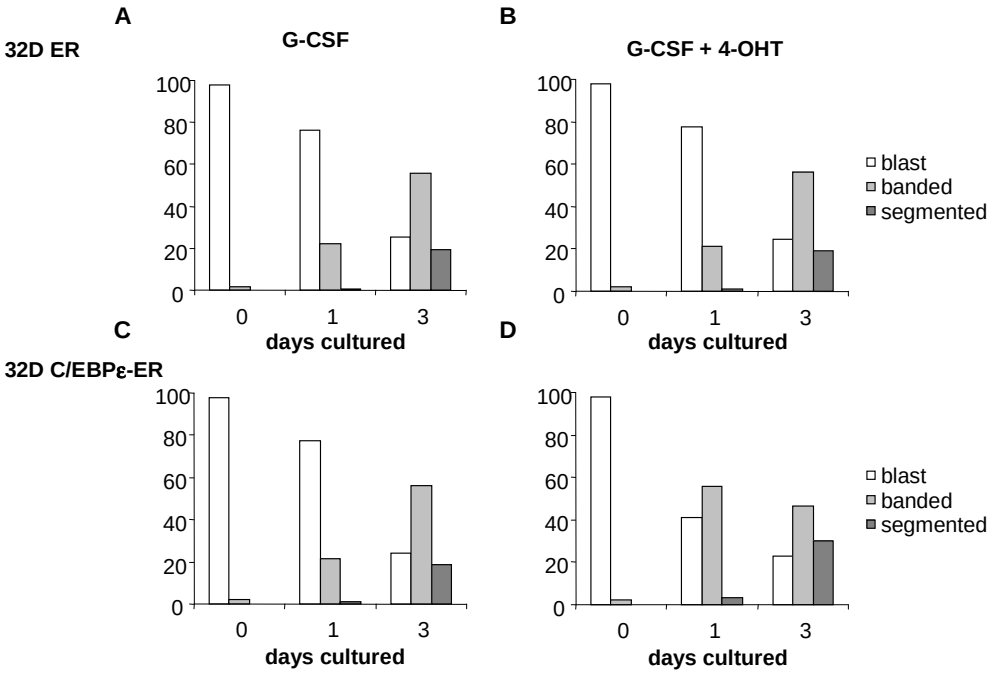

Figure 4. C/EBPε enhances granulocytic morphology during G-CSF induced granulocytic differentiation. Cytospins were made, stained with the May-Grünwald Giemsa solution and determined by microscopy. The percentage of blast, banded, segmented or highly-segmented granulocytes is shown as an average of two individual experiments.

Effect of Inducible C/EBPε Expression on Proliferation of G-CSF-Induced 32D Cells

Induction of differentiation is associated with growth arrest and thus reduced proliferation. Therefore we examined whether the enhanced differentiation of 32D C/EBPε-ER in the presence of G-CSF and 4-OHT also resulted in a more pronounced reduction of the proliferation. To study the proliferation, 32D C/EBPε-ER and 32D-ER cells were seeded in IL-3 or G-CSF in the presence or absence of 4-OHT and viable cell counts were made daily (Figure 5). The 4-OHT did not alter the growth of 32D-ER and 32D- C/EBPε-ER cells in the presence of IL-3. In the presence of G-CSF there is a decrease in the proliferation as shown before [44]. We did not observe a difference in proliferation of 32D-ER cells cultured in G-CSF and 4-OHT. However, 32D- C/EBPε-ER cells exposed to G-CSF and 4-OHT showed a small but significant decrease of proliferation compared to cells grown in G-CSF alone.

It is tempting to speculate about the reduced proliferation upon induction of C/EBPε. C-myc is one of the critical regulators of cellular proliferation [45-47]. C-myc is expressed in proliferating cells, whereas C/EBPs are expressed in differentiated cells [47; 48]. Over-expression of c-myc blocks differentiation in many cell types [49-51]. In 32D cells, over-expression of c-myc also blocks differentiation by G-CSF, but the underlying molecular mechanism of this effect is not well understood [52]. It has been shown that c-myc represses C/EBPα expression and it has been demonstrated that C/EBPα negatively regulates c-myc transcription [53; 54]. Recently it was demonstrated that c-myc inhibits C/EBPε transcription [43]. C/EBPα was previously found to inhibit proliferation of adipocytes and hepatocytes [17; 55]. In addition, hepatocytes from newborn C/EBPα-deficient mice display increased proliferative activity [56]. C/EBPα may inhibit the proliferation via interaction with E2F or with cdk2 [54; 57-59]. Therefore, an intriguing possibility is that C/EBPε may also inhibit proliferation by binding to c-myc, p21 or other CDK inhibitors.

Another interesting point is the signals delivered by the G-CSFR. There are two models which explain the role of cytokines in hematopoiesis. In the stochastic model the only role of cytokines is to support survival of the progenitors that express its cognate receptor on their surface [60; 61]. These receptor-expressing cells are destined to execute an intrinsically preset differentiation program. In contrast, the instructive model suggests active roles for lineage-specific cytokines in directing various differentiation processes [62-64]. We show that G-CSF signalling is necessary for induction of myeloid differentiation by C/EBPε. Induction of C/EBPε with 4-OHT in the presence of survival factor IL-3 was not enough to induce differentiation. Therefore it seems that the G-

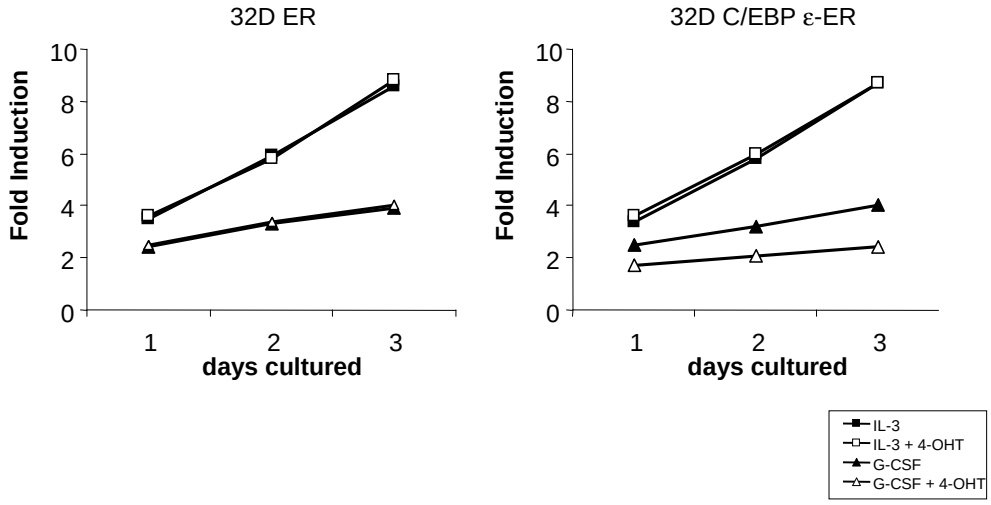

Figure 5. C/EBPε inhibits proliferation during granulocytic differentiation of 32D cells. 32D C/EBPε-ER and 32D-ER cells were seeded at 1 x 10^5 cells/mL in IL-3 or G-CSF, in the presence or absence of 4-OHT, and viable cell counts were made daily. Results are expressed as fold increase in cell numbers compared with day 0 as an average of three individual experiments.

CSF-R provides more than simply survival signals to enable lineage development, indicating that inductive signals are involved in lineage differentiation. The G-CSF-R activates several tyrosine kinases as well as the JNK-pathway, STAT proteins, and components of the MAPK-pathway [65; 66]. It is intriguing to know which signals of the G-CSF-R are involved in the differentiation. It is possible that the signals presented by the G-CSF-R regulate C/EBPε activity. However, also signals involved in other differentiation inducing pathways can be provided. The first tyrosine of the G-CSF receptor was shown to be involved in the expression of C/EBPε [43]. Further experiments with the different tyrosine mutated G-CSF receptors will give new information about the signals involved in C/EBPε expression and activation and other signals involved in granulocytic differentiation.

Taken together, we showed that activation of the G-CSF-R together with the expression of C/EBPε enhances differentiation of 32D cells. Because we used a tamoxifen inducible system we were able to show that expression of C/EBPε directly enhances the differentiation in the presence of G-CSF. Expression of C/EBPε-ER decreases proliferation of differentiating 32D cells compared to differentiation induced by G-CSF alone. This indicates that C/EBPε is involved in the growth arrest of differentiating granulocytes. Both G-CSF and C/EBPε are necessary in granulocytic differentiation in 32D cells. Further investigation is necessary to elicit the role of G-CSF-R signalling in activation of C/EBPε and other signals involved in the differentiation of granulocytes.

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Chapter 7

General discussion

Contents

General Discussion

Eosinophils play an important role in the pathogenesis of allergic diseases, such as allergic asthma. Eosinophils normally constitute only a few percent of circulating leukocytes. Increased numbers of eosinophils are found in peripheral blood, tissues, and bronchoalveolar lavage of allergic asthma patients [1; 2]. In the bone marrow multipotent stem cells can differentiate towards mature eosinophils, which is associated with the expression of specific genes, coding for e.g. the granule proteins. A highly organised network is required to coordinate these events and to maintain steady state levels of mature cells, as well as to stimulate the rapid production of specific cell types when needed. This thesis has focused on the transcription factors involved in the regulation of maturation of human eosinophils. An ex-*vivo* differentiation model was developed to study eosinophilopoiesis, and the role of transcription factors during this process was investigated. Our findings are discussed here with respect to hematopoietic cell fate decisions.

The maintenance of adequate numbers of terminally differentiated cells during health and disease is ultimately regulated by the continuous self-renewal, controlled proliferation, and differentiation of hematopoietic stem cells [3; 4]. Closely regulated expression of cytokines, growth factors, receptors and transcription factors coordinate lineage development. Cytokines and growth factors are soluble glycoproteins released by cells, which exerts their activities by high affinity binding to a specific receptor [5]. Some cytokines promote the growth and survival of progenitors of multiple lineages, such as IL-3 and SCF. Other cytokines are more restricted in their actions and support the development of a particular lineage, such as G-CSF and TPO, which are involved in the development of granulocytes and megakaryocytes respectively [6; 7].

Transcription factors

1. Lineage specificity: lineage-specific transcription factors

During hematopoiesis, cells undergo maturation resulting in a coordinated, cell-specific gene-expression pattern. The pattern of gene-expression within a cell can be established by cell-specific transcription factors that mediate the net effect of the variety of proliferation and differentiation signals which impinge on it. One convenient way to coordinate the expression of many individual genes in a terminally differentiated cell is through the guidance of a few cell-specific transcription factors. Several examples of this situation have been published. A clear example has been found in skeletal muscle lineage development. There are four myogenic basic helix-loop-helix (bHLH)

factors MyoD, myogenin, Myf5, and MRF 4, which are specifically expressed in muscle cells. Together with the more general expressed MEF2 these four bHLH factors act cooperatively to regulate the expression of skeletal muscle-specific genes [8]. Expression of one of these factors into non-muscle cells results in the activation of the entire program of muscle development. Expression of the transcription factors involved in hematopoiesis is not restricted to one lineage, indicating that the processes actually governing hematopoietic cell differentiation are undoubtedly more complex.

2. Lineage specificity: unique combinations of transcription factors

The processes leading to differentiation of stem cells to mature hematopoietic cells are more complex than simply lineage specific expression of a transcription factor. This because transcription factors involved in myelopoiesis are not restricted to myeloid cells. Rather than being controlled by single master regulators, lineage-specific gene expression appears to depend on the combination of transcription factors expressed in a particular lineage. PU.1 and C/EBPα cooperate to activate the G-CSF receptor; AML-1 and c-myb synergize in the activation of the myeloperoxidase gene; PU.1 cooperates with C/EBPα, c-myb and AML-1 in the activation of the neutrophil elastase gene promoter.

We found cooperative regulation of the common β chain of the IL-5R by C/EBPα and PU.1 (chapter 4). Interestingly, we also located an intronic region which was involved in the regulation of the βc promoter. This region from +520 to +640 contains a putative GATA-1 binding site at +550. The three regions are involved in gene regulation. Mutation of this GATA-1 element will determine whether it is indeed responsible for the enhancer activity of this region. The putative regulation of the βc promoter by GATA-1 is an interesting concept. The expression of the β-subunit is not restricted to one cell type, but is expressed by all myeloid cells. An explanation for this might be that specific complexes define lineage and differentiation stages. Starting with gene regulation by a protein complex in an early progenitor, factors can be replaced, resulting in complexes that specify different cell types and differentiation stages. It is possible that in neutrophils, expression of the βc is regulated by PU.1 and C/EBP proteins, whereas in eosinophils C/EBP and GATA-1 control expression (figure 1). In addition, since the common β chain is already expressed in early progenitors, regulation must be subject to transcription factors already expressed early during differentiation. Both PU.1 and GATA-1 are expressed in early progenitors [9; 10]. However, PU.1 and GATA-1 are known to inhibit each others activity [11-14]. Probably other transcription factors are involved in gene regulation of the βc in early progenitors.

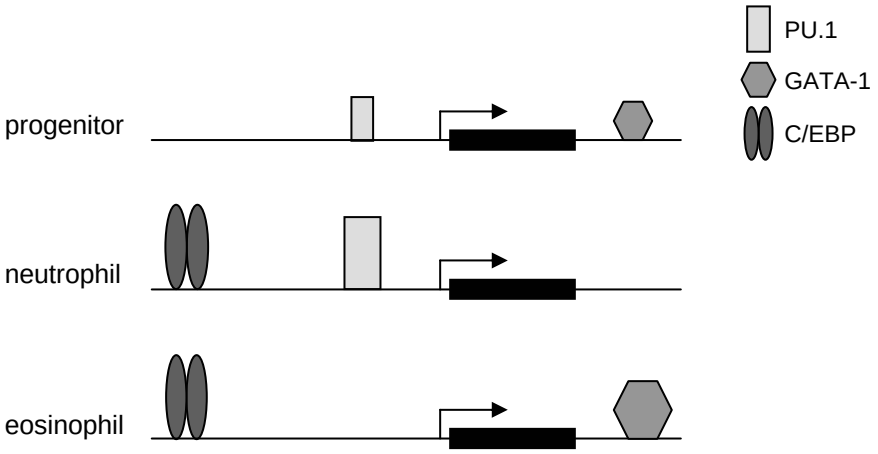

Figure 1. Regulation of the βc gene during hematopoiesis Is the common β chain different regulated during hematopoiesis? Regulation of the βc gene occurs in different cell types. We found that different regions of the promoter are involved in the activation. Different transcription factor complexes can be involved in the different cell types during differentiation. C/EBP and GATA-1 might regulate expression in eosinophils and PU.1 and C/EBP probably activates the promoter in neutrophils. GATA-1 and PU.1 are both expressed in progenitors, but at a low level.

2.1 Relative amounts of transcription factors

Beside the presence of a transcription factor, the relative amount of specific protein regulators appears also to be involved in lineage decisions. Low amounts of a transcription factor present in a progenitor results in different cell fate decision compared to progenitors expression a high amount of the transcription factor. Interesting is to investigate how that amount of the transcription factors is controlled. PU.1 is required for the proper generation of both myeloid and lymphoid lineages. The choice of monocyte or B-lineage specific gene expression is mediated by different concentrations of PU.1. High concentrations direct progenitors towards monocytes, whereas low concentrations promote the B-lymphocytes.

An example of gene activation regulated by relative amount of transcription factor is provided by the eosinophil-specific EOS47 gene, the avian ortholog of the mammalian melanotransferrin. C/EBPα and Ets-1 were found to cooperate in both the binding to, and activation of, the EOS47 promoter [15]. Interestingly, while low levels of GATA-1 enhanced promoter activation by C/EBPα and Ets-1, high levels led to a repression. This repression was further enhanced by FOG-1, while GATA-1 on its own has little effect [16]. This positive and negative regulation of the EOS47 promoter closely mimics the distribution of the proteins in the hematopoietic system (figure 2).

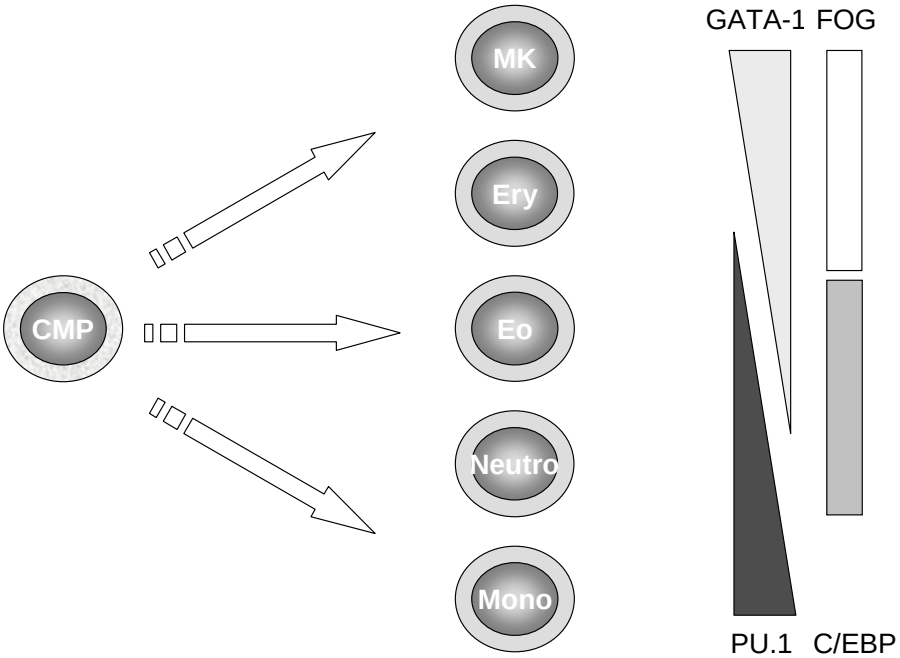

Figure 2. Distribution of transcription factors in hematopoietic cells GATA-1 is highly expressed in erythrocytes and thrombocytes and at a moderate level in the eosinophil lineage. PU.1 expression is more pronounced in the neutrophil and monocyte lineage. FOG-1 is expressed in erythrocytes and thrombocytes. C/EBP proteins are expressed in eosinophils and neutrophils.

2.2 Antagonism between different lineage-specific transcription factors

Over the last years the occurrence of antagonism between different lineage-specific transcription factors has gained much interest. Transcription factors expressed simultaneously can exert inhibitory effects on lineage programs by directly antagonising the action of opposing transcription factors. The direct physical interaction and cross-antagonism between GATA and PU.1 has been demonstrated [11-14]. In addition, the antagonistic relationship between the transcription factor C/EBPβ and FOG in avian models has been reported at the transcriptional level [16].

It seems that the common myeloid progenitor (CMP) is expressing both GATA-1 and PU.1 at similar low levels [9; 10]. Probably they are functionally antagonising each other, which results in absence of transcription of their lineage-specific target genes. When the abundance of one of the transcription factors increases, and exceeds that of the other transcription factor, this results in the activation of target genes, thereby directing cell fate. A positive auto-regulatory feedback loop may then further increase the expression of the transcription factor (see figure 3).

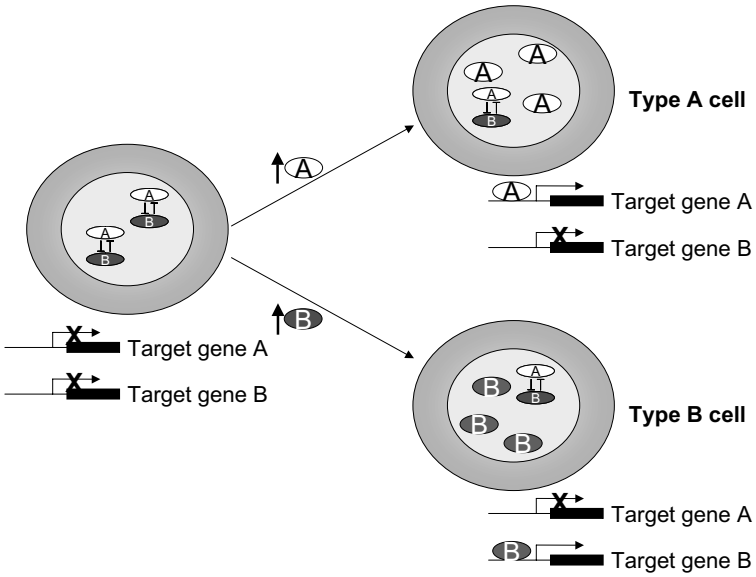

Figure 3. Cross-antagonistic model of lineage determination In early progenitors, lineage-specific transcription factors representing opposing lineages (A and B) are present at similar low levels. Because they cross-antagonise each other neither of their target genes is expressed. When the abundance of one of the lineage-specific transcription factors increases and exceeds the other, target genes are activated.

During eosinophil differentiation there is cross-antagonism between C/EBP proteins and FOG. This cross-antagonism between C/EBPβ and FOG has been shown for both the avian EOS47 gene and the mammalian granule major basic protein (MBP). The interaction of GATA-1 and FOG is necessary for terminal erythroid maturation and thrombopoiesis. Prevention of these pathways is possibly necessary in eosinophil commitment.

A moderate level of GATA-1 in the cell and the presence of C/EBP proteins are important for eosinophil lineage determination [16-18]. On the other hand PU.1 is important for eosinophil differentiation because PU.1 knockout mice lack eosinophils. There is some evidence that eosinophils express small amounts of PU.1, but levels are very low compared to less mature cell lines (S.J. Ackerman, unpublished result). Therefore it seems that PU.1 expression is induced during the early stages of eosinophilopoiesis, but later during differentiation there is an inhibition of PU.1. The presence of GATA-1 proteins in mature eosinophils suggests an antagonism between GATA-1 and PU.1 in at some point of the development of eosinophils.

3. Regulation by multiple promoters

As previously mentioned some lineage-specific proteins, such as receptors, are already expressed in early progenitors even though these progenitors lack lineage specific transcription factors. An explanation for this might be the involvement of unique combinations of widely expressed or even ubiquitous transcription factors and lineage specific factors. The c-Jun oncoprotein forms a heterodimer with the c-Fos protein to form the AP-1 transcription factor, which has been shown to be involved in cell proliferation and differentiation [19; 20]. C-jun is an early response gene in differentiating myeloid cells and its expression is upregulated during differentiation [21; 22]. C-jun can also physically interact with PU.1 and this interaction is important during myeloid differentiation [23; 24]. Interestingly in chapter 3 we found the involvement of an AP-1 site in the regulation of the eosinophil specific IL-5 receptor alpha-subunit. The IL-5Rα promoter was described in 1995, and was found to be active in the acute myeloid leukemia cell line AML 14, but not in the pro-myelocytic HL-60 cell line [25]. Interestingly, promoter activity was enhanced in eosinophil committed cells. An essential transcription factor binding site was found at 421 bp upstream of the transcriptional start site. Recently it was reported that a ubiquitously expressed protein, named regulatory factor X (RFX), binds to this site and regulates transcription of the IL5Rα gene [26]. The crucial AP-1 site reported in chapter 3 is located directly upstream of the binding site of RFX. A second promoter (P2) was also identified suggesting that IL-5Rα gene expression might be regulated by multiple promoters [27]. Promoter activity of this second promoter

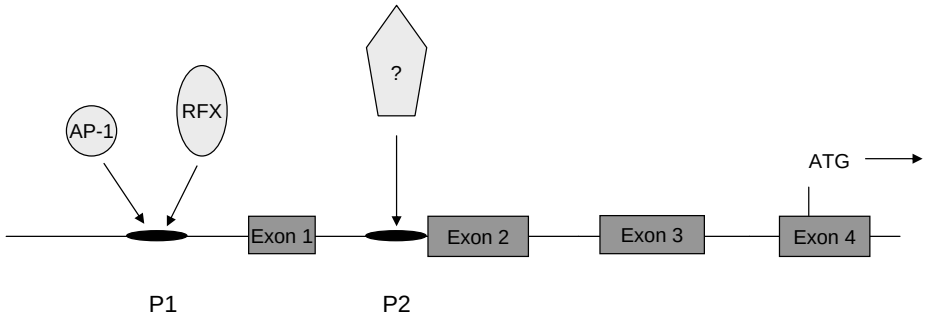

Figure 4. Regulation of the IL-5Rα gene by 2 promoters Regulation of IL-5Rα expression is under control of 2 promoters. The first promoter which is activated by the general expressed AP-1 and RFX factors and a second promoter which seems more involved in terminal differentiated cells. The protein (complex) binding to this second promoter is unknown.

is only regulated in an eosinophil committed HL-60 cell line. In this promoter a 6 bp element is required for promoter activity and binds to a protein (complex) specific for eosinophilic cells. The regulation of other tissue-specific and developmentally regulated genes has been shown to be dependent on multiple promoters [28-30]. It is possible that during eosinophil maturation, an alternative use of the promoters may determine stage specific expression of the IL-5Rα gene (figure 4). Early during differentiation IL-5Rα expression is regulated by P1, which is regulated by general transcription factors. Although this regulation by general transcription factors is cell specific, since IL-5Rα is limited to early progenitors, basophils and eosinophils. Therefore there must be a specific regulation in early progenitors, although this regulation remains to be solved. The second promoter, P2, becomes more important during later stages of eosinophil development, probably by more lineage-restricted proteins. However, the functional site of the second promoter has no match for known transcription factors. It would be interesting to know what transcription factor (complex) is involved in the activation of the second promoter. Another interesting point is the regulation by these two promoters. Is regulation of IL-5Rα in early progenitors under the control of the first promoter and later on directed by the second promoter or do they work together in more differentiated cells? Further investigation is necessary for the differential regulation of the IL-5Rα during eosinophilopoiesis.

4. Consequences for eosinophilopoiesis

In the lineage commitment of the common myeloid progenitor (CMP) to the different mature cell types, the transcription factors PU.1, GATA-1, FOG and C/EBPs seem to be involved. Due to the complexity of the role of transcription factors during hematopoietic differentiation, several hypotheses can be proposed. First a classical linear model, in which the relative expression of the proteins is regulating lineage determination. The expression levels of PU.1 and GATA-1 decide the fate of the CMP towards the different progenitors. During differentiation, the expression of lineage specific proteins determines lineage decisions. Expression of C/EBP in the GM-progenitor results in production of neutrophils, whereas expression of ICSBP favours monocytic differentiation. Eosinophils are formed by moderate expression of GATA-1, and later on by the expression of C/EBP proteins. These progenitors have one decision point and become mature cells in a linear fashion (figure 5 model 1). This is the classical view of hematopoiesis. Here we propose an alternative hypothesis (figure 5 model 2). In this model we assume that the differentiation of cells is more complex and that there might be different ways to for a cell to differentiate into an eosinophil. Akashi *et al*. showed that both CMPs and Granulocyte/Monocyte Progenitors can generate eosinophil colonies [31]. Very recently it was shown that $CD34^+$ cells transduced with GATA-1, committed towards neutrophils, express eosinophil markers [32]. In our $CD34^+$ culture we also observe eosinophils when cultured under neutrophil conditions and neutrophils when cultured under eosinophil conditions (unpublished data). Taken together, this might indicate that the bifurcation between eosinophils and neutrophils could be late during differentiation.

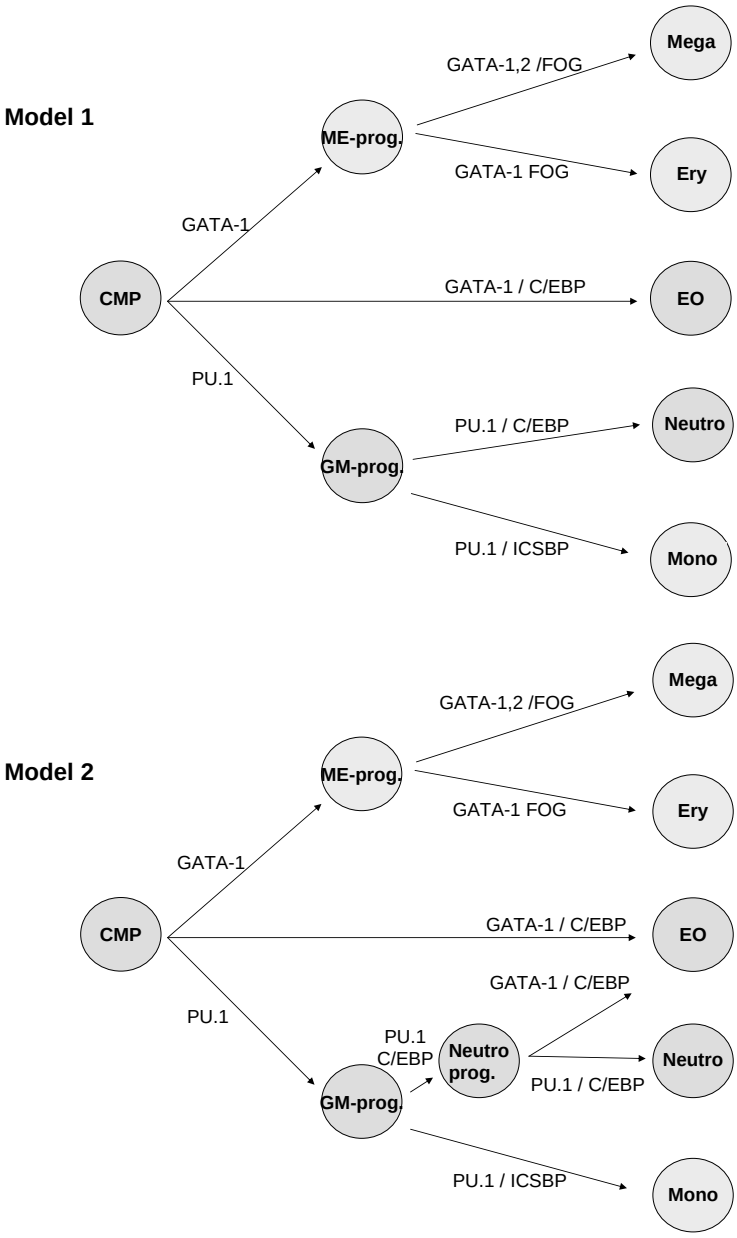

Figure 5. Two Models for myeloid differentiation Model 1 is a classical linear model. In this model the expression of the proteins is regulating lineage determination. In model 2 we assume that the eosinophil differentiation is not a linear process, but that there are different ways. In both of these models we did not include the basophil, because the role of transcription factors in basophils has not been well investigated yet. Although a progenitor for basophils and eosinophils has been reported the identity of the basophil precursor is not clear. CMP: common myeloid progenitor. ME-prog.: megakaryocyte and erythrocyte progenitor. Eo-prog.: eosinophil progenitor. GM-prog.: granulocyte and monocyte progenitor. Mega: megakaryocyte. Ery: erythrocyte. EO: eosinophil. Neutro: neutrophil. Mono: monocyte

5. From cytokine to transcription factor

Transcription factor function is modulated by numerous influences including signals delivered by cytokines and growth factors. In contrast to the role of transcription factors in hematopoiesis, surprisingly little is known about the role of signalling molecules in this process. Cytokines are pleiotropic; they can act on different cells and induce a wide range of responses [5]. These multiple biological effects can be explained by the range of signalling components and transcription factors present in the target cells at the time of stimulation with the cytokine. Therefore it is interesting to have a closer look at the role of cytokines on eosinophil differentiation.

Several studies have demonstrated that $CD34^+$ cells cultured in the presence of IL-5 were committed to the eosinophil lineage and were capable of forming eosinophil colonies [33] indicating that IL-5 promotes growth and differentiation of stem cells to mature eosinophils [34; 35]. Binding of IL-5 to its receptors rapidly induces activation of multiple intracellular signalling pathways, including the Ras-Raf-ERK, the JAK/STAT, the phosphatidylinositol 3-kinase (PKB), and the JNK/SAPK and p38 signalling pathways [36; 37]. Transcriptional initiation is regulated by altering the properties of DNA-binding proteins, such as (de)phosphorylation, DNA-binding activity and nuclear localization. Several studies have suggested a potential role of phosphorylation in the regulation of C/EBPα activity. In granulocytes activation of the Ras pathway can induce phosphorylation of C/EBPα at several sites (S-248, S-277, and S-299) by Protein Kinase C (PKC) [38; 39]. Phosphorylation of C/EBPα results in an enhanced transactivation, for example of the G-CSF receptor promoter. In addition, C/EBP proteins have been demonstrated to synergistically activate transcription the oncostatin M promoter with STAT5, which itself is activated by IL-5 induced signalling [40].

How the signalling pathways activated by IL-5 could be involved in the regulation of eosinophil differentiation is still intriguing. In granulocytes, G-CSF induces the expression of C/EBPα and C/EBPε during differentiation, although the factors regulating this expression remain unclear ([41], chapter 6). Whether IL-5 is involved in the regulation of C/EBP expression is not known. Expression of C/EBPε alone is not sufficient for neutrophilic differentiation, indicating that other signals delivered by the G-CSF-R are involved in this process. Since in neutrophils G-CSF signalling is necessary for differentiation, this might indicate that in eosinophil additional IL-5 induced signals are also involved in the regulation of maturation. Additional signals from the IL-5R involved in eosinophil differentiation should be specific. This could be potentially established by signals generated by the eosinophil specific alpha-subunit.

6 Stochastic versus inductive

There is scarce data available concerning the molecular mechanisms regulating expression of key transcription factors, which leads to controversy about cell fate decisions in hematopoiesis. For example, do cytokines instruct target cells to commit to particular blood lineages (the inductive model) or do they simply "select" cells that already have chosen a particular path that includes the expression of the lineage restricted receptors (the stochastic model).

The stochastic model implies that lineage decisions in hematopoiesis arise by chance and that growth factors act simply to promote the survival and growth of cells in which the decision has already been made. Stem cell commitment might arise through the co-expression of combinations of lineage specific transcription factors and hematopoietic growth factor receptors. At an early stage of commitment any growth factor may suffice because the transcription factors determine the cellular phenotype. Subsequently, the transcription factors will increase the probability of expression of lineage-restricted growth factor receptors, further reinforcing the commitment event.

The inductive model suggests that the lineage-specific genes are expressed in response to an appropriate external stimulus. In response to that stimulus, they must rapidly increase the transcription of lineage specific genes. Such a mechanism can only operate if most of the genes required for hematopoietic lineages are in open chromatin in stem cells and available for transcription. Indeed, Hu *et al* found evidence of a so-called promiscuous phase of multi-lineage gene expression in which myeloid and erythroid lineage genes, including growth factor receptors, are co-expressed [10].

Many lineage-specific transcription factors are involved in gene regulation of lineage specific receptors, e.g. C/EBPα and PU.1 in G-CSF-R regulation [41]. In chapter 5, we showed that inhibition of C/EBP transcription factors leads to the down-regulation of the βc. In chapter 6 we show that for granulocyte differentiation both C/EBPε and G-CSF are involved. G-CSF is able to induce granulocytic differentiation by induction of C/EBPε. However, C/EBPε needs G-CSF stimulation to rapidly induce granulocytic differentiation. This stimulus can both activate C/EBPε and provide additional signals involved in granulocytic differentiation.

Are cytokines responsible for lineage commitment or are transcription factors the basis of lineage determination? Evidence for the role of cytokines and cytokine receptors in hematopoiesis has come from studies in knockout mice. The IL-5$^{-/-}$, IL-5Rα$^{-/-}$, $\beta_c^{-/-}$ and β_c/IL-3 double knock-out mice retain lower, but detectable levels of eosinophils [42-44]. This indicates that in the absence of the specific cytokine, differentiation might occur, albeit at a low efficiency. On the other hand C/EBPα and C/EBPε knockout mice do not have mature eosinophils demonstrating that the lack of transcription factors involved in the regulation of differentiation leads to a block of eosinophil production. In chapter 6 we show that over-expression of C/EBPε could induce granulocytic differentiation, however in the presence of both C/EBPε and G-CSF signalling, differentiation occurs more rapidly.

We propose a model for the stochastic and inductive processes during hematopoiesis. The initial steps of stem cell differentiation appear to involve the stochastic expression of specific transcription factors and subsequent induction of lineage-restricted receptors. Therefore these cells are able to respond to external signals from cytokines. Cytokines than act to expand the population of lineage committed progenitor cells. Therefore in the absence of the specific cytokine, terminal differentiation might occur but at a very low efficiency (figure 6).

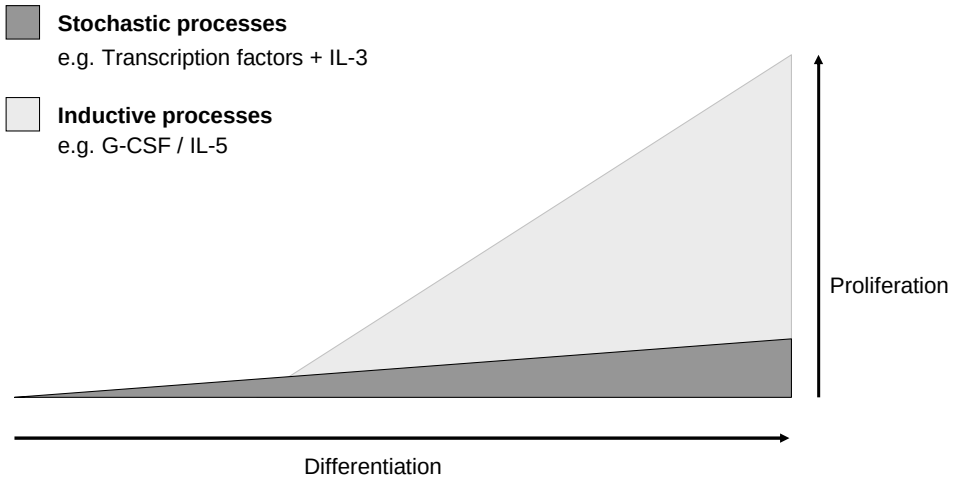

Figure 6. Stochastic or Inductive In this model we show that differentiation is a result of stochastic processes in which the programmed cell differentiate and the “inductive model” is involved in the proliferation during terminal differentiation.

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Nederlandse Samenvatting

Hematopoiesis

Een volwassen mens heeft ongeveer vijf liter bloed waarin zich miljarden cellen bevinden. Deze cellen zijn onder te verdelen op basis van hun verschillende functies: witte bloedcellen (afweer tegen ziekteverwekkers), rode bloedcellen (zuurstoftransport) en bloedplaatjes (stolling van het bloed). Bloedcellen leven niet lang en daarom moeten er dagelijks miljarden cellen worden bijgemaakt. Deze massa productie van bloedcellen (hematopoiesis) vind plaats in het beenmerg, de holten van onze botten. De verschillende soorten cellen ontstaan uit stamcellen. Het belangrijkste kenmerk van stamcellen, naast het feit dat ze zich kunnen ontwikkelen tot de verschillende bloedcellen, is dat ze zichzelf kunnen vernieuwen. Hierdoor zijn er gedurende het hele leven cellen aanwezig die zorgen voor de continue aanmaak van nieuwe bloedcellen. Een stamcel kan zich via een tussenfase van voorlopercel ontwikkelen tot elk gewenst type bloedcel (zie hoofdstuk 1, figuur 3). Dit proces van ‘volwassen worden’ van een cel wordt differentiatie genoemd. Dit gecompliceerde proces wordt beheerst door mechanismen die ervoor zorgen dat juist díe bloedcellen worden aangemaakt waaraan de behoefte het grootst is en dat de hoeveelheden bloedcellen in het bloed constant blijven. Hierbij spelen hematopoietische groeifactoren, regulerende eiwitten, een rol. Ook bepaalde bindweefselcellen in het beenmerg, de steuncellen, vervullen hierbij een functie.

Eosinofiele granulocyten

Eosinofiele granulocyten (eo’s) zijn witte bloedcellen die een korte periode in de bloedbaan aanwezig zijn, maar dan uittreden naar het weefsel. Mede daardoor maken eo’s maar een klein deel van de celpopulatie in het bloed uit. Eo’s danken hun naam aan het feit dat ze voor het eerst werden gekleurd met de kleurstof eosin. Eosin kleurt de basische eiwitten in de korrels (granula) van de cel. Deze korrels zijn een belangrijk kenmerk van eo’s, evenals hun tweelobbige kern.
Het verlaten van de bloedbaan door eo’s is een goed gereguleerd proces en vindt plaats in verschillende stappen . In de weefsels aangekomen kunnen eo’s worden geactiveerd, dit leidt tot het uitscheiden van schadelijke eiwitten, zuurstofmetabolieten en ontstekingsmediatoren .
Eosinofiele granulocyten spelen een positieve rol in infectie’s met wormen, omdat de basische eiwitten in de korrels parasieten kunnen vernietigen. Hierdoor worden vervelende infectie’s met parasieten voorkomen. Aan de andere kant spelen eo’s een belangrijke, hetzij negatieve rol, in de ontwikkeling van allergische ziekten, zoals allergisch astma. Patiënten met allergisch astma reageren op stoffen uit de omgeving die normaal gesproken niet schadelijk zijn voor het lichaam, bijvoorbeeld huisstofmijt, kattenharen of graspollen. De door eosinofiele granulocyten uitgescheiden stoffen beschadigen weefsel in de longen, met benauwdheid en chronische ontstekingen tot gevolg.

Groeifactoren en receptoren

Cellen kunnen communiceren met hun omgeving, dit gebeurd met behulp van verschillende moleculen. Een type van die moleculen zijn de cytokinen. Cytokinen zijn eiwitten die worden

uitgescheiden en verschillende functie's hebben, zoals overleving, activatie en differentiatie. Er zijn veel verschillende cytokinen, sommige met specifieke functie's andere met overlappende functie's. De cytokinen aan de buitenkant van een cel kunnen signalen afgeven aan de cel door middel van receptoren. Deze eiwitten hebben een gedeelte buiten de cel om cytokinen te kunnen binden en een gedeelte binnen de cel om signalen door te kunnen geven aan andere eiwitten. De cytokine, interleukine 5 (IL-5) speelt een belangrijke rol in de groei, differentiatie en activatie van eo's. De receptor voor IL-5 bestaat uit twee componenten. Een β-keten die ook wordt gebruikt voor de receptoren voor de cytokinen interleukine-3 en GM-CSF (granulocyte macrophage colony stimulating factor) en een α-keten die specifiek is voor de IL-5 receptor en daardoor alleen op eo's voorkomt.

Transcriptie

Iedere cel maakt verschillende eiwitten voor verschillende doeleinden. Sommige van die eiwitten zijn voor iedere cel van belang en komen dan ook in alle cellen voor. Andere eiwitten zijn specifiek voor één bepaalde cel. Wat bepaalt dat in een bepaalde cel een eiwit wel voorkomt en in een andere niet? Op het humane genoom (het complete DNA van de mens) liggen ongeveer 30.000-35.000 genen. Deze genen maken slechts circa 1-2 procent uit van het complete genoom. De rest van het DNA bevat regulerende delen (promotor) ofwel delen met een nog onbekende functie. Een gen codeert voor één of meerdere eiwitten. De eerste stap om van een gen een eiwit te maken noemen we transcriptie. De transcriptie van een gen wordt gereguleerd door de binding van transcriptie factoren op specifieke plaatsen in de promotor. De promotor ligt meestal voorafgaand aan een gen op het genoom en is als het ware de regulator van het gen. Sommige transcriptie factoren zijn zelf actief, andere hebben de hulp van andere nodig voor volledige activatie. De aanwezigheid en activiteit van transcriptie factoren bepalen dus welke eiwitten er in een cel voorhanden zijn. Tijdens de vorming van nieuwe bloedcellen zijn transcriptie factoren dan ook van belang omdat de aanwezigheid van de verschillende eiwitten bepaalt wat voor een soort cel er ontstaat. Er zijn verschillende transcriptie factoren bekend die een rol spelen tijdens de hematopoiesis.

Dit proefschrift

Het eiwit EDN (eosinophil-derived neurotoxin) is te vinden in de specifieke korrels van eosinofiele granulocyten. In hoofdstuk 2 wordt de regulatie van transcriptie van EDN beschreven. Verschillende leden van de C/EBP familie van transcriptie factoren zich kunnen binden aan de promotor van het EDN gen. Deze binding van C/EBP eiwitten is van belang voor de transcriptie regulatie van EDN. Vernietiging van deze bindingsplaats in de promotor resulteert in een afname van de activiteit.

In hoofdstuk 3 wordt een gebied in de promoter van de α-keten van de IL-5 receptor geïdentificeerd die betrokken is bij de transcriptie regulatie van dit eiwit. Dit gebied bindt verschillende familieleden van de AP-1 familie van transcriptie factoren. Deze AP-1 bindingsplaats reguleert

samen met een eerder geïdentificeerde bindingsplaats de transcriptie van de α-keten in eosinofiele granulocyten.

Het is al bekend dat de β-keten wordt gereguleerd door PU.1, een lid van de Ets transcriptie factor familie die specifiek voorkomt in hematopoietische cellen. In hoofdstuk 4 wordt een tweede transcriptie activatie gebied geïdentificeerd in de promotor van de β-keten. Dit gebied bestaat uit twee elementen die zowel C/EBPα, C/EBPβ, als C/EBPε kunnen binden. Verwijdering van dit gebied of mutatie van een van de twee C/EBP bindende elementen resulteert in een verlies van promotor activiteit. Verder wordt een samenwerking in de activatie van transcriptie tussen PU.1 en C/EBP eiwitten aangetoond.

In hoofdstuk 5 wordt de rol van C/EBP transcriptie factoren onderzocht tijdens de differentiatie van humane $CD34^+$ cellen naar eosinofiele granulocyten. Hiervoor wordt gebruik gemaakt van een differentiatie modelsysteem van stamcel naar eosinofiele granulocyt. $CD34^+$ hematopoietische voorloper cellen worden geïsoleerd uit navelstrengbloed en onder invloed van verschillende cytokinen, voornamelijk IL-5, kunnen, we een differentiatie bereiken naar 70% eosinofiele granulocyten. In dit systeem is de C/EBPα expressie hoog gedurende de beginfase van de differentiatie en neemt af later tijdens de differentiatie. In tegenstelling tot C/EBPα, neemt de expressie van C/EBPε toe tijdens de differentiatie. Een dominant negatieve variant van de C/EBP familie werd in de differentiërende cellen tot expressie gebracht door middel van retrovirale infectie. Remming van de C/EBP familie tijdens differentiatie van eosinofiele granulocyten resulteerde in een afname van de groei en in een blokkade van de differentiatie. Bovendien was de expressie van beide ketens van de IL-5 receptor drastisch verlaagd.

Zoals IL-5 betrokken is bij de differentiatie van eo's reguleert G-CSF (Granulocyte colony-stimulating factor) de differentiatie van neutrofiele granulocyten. Het is bekend dat ook in neutrofiele granulocyten C/EBP eiwitten een rol spelen. In hoofdstuk 6 wordt de rol van de C/EBPε transcriptie factor in the G-CSF geïnduceerde granulocyte differentiatie onderzocht. Hiervoor werd gebruik gemaakt van een induceerbaar systeem, dat zorgt voor een snelle en directe activatie van C/EBPε. Expressie van C/EBPε tijdens G-CSF geinduceerde differentiatie resulteerde in een versnelling van de differentiatie en een afname van de groei. Stimulatie van voorloper cellen met G-CSF resulteert in de vorming van granulocyten, echter dit proces verloopt sneller in de aanwezigheid van C/EBPε. Het is echter niet bekend via welke eiwitten de signalen van de G-CSF receptor naar C/EBP eiwitten worden doorgegeven.

Uit het onderzoek beschreven in dit proefschrift blijkt dat verschillende transcriptie factoren betrokken zijn de vorming van eiwitten in eosinofiele granulocyten. De rol van C/EBP factoren in eo-differentiatie is van groot belang. Remming van deze eiwitten zorgt ervoor dat er geen eo's ontstaan, maar C/EBP eiwitten komen ook in andere cellen voor. Dit betekent dat alleen de aanwezigheid van C/EBP eiwiitten niet bepalend is voor de vorming van eo's. Het lijkt er dan ook op dat een complex netwerk van transcriptie factoren en cytokinen bepaald welke cel er gevormd wordt.

Hierbij wil ik alle mensen bedanken
voor de ontzettend leuke tijd, alle gezelligheid
de steun en adviezen van de afgelopen 4 jaar

Curriculum Vitae

De schrijfster van dit proefschrift werd op 12 juli 1973 geboren in Arnhem. Na het behalen van haar VWO diploma aan het Thomas à Kempis College te Arnhem werd begonnen met de studie Medische Biologie aan de Universiteit Utrecht. In april 1998 werd het doctoraal examen gehaald met als bijvak Hematologie / Oncologie (Dr. G.J.J.C. Boonen en Prof. Dr. J.J. Sixma, Universiteit Utrecht) en als hoofdvak Moleculaire Biologie (Dr. R. P. de Groot en Prof. Dr. L. Koenderman, Universiteit Utrecht). In juni 1998 begon zij als AIO bij de afdeling Experimentele Longziekten van het Universitair Medisch Centrum te Utrecht onder begeleiding van Prof. Dr. Leo Koenderman en Dr. Rolf P de Groot.

List of publications

T.B. Van Dijk, **B. Baltus**, E. Caldenhoven, H. Handa, J.A.M. Raaijmakers, J.-W.J. Lammers, L. Koenderman, and R.P De Groot. Cloning and characterisation of the human IL-3/IL-5/GM-CSF receptor βc gene: regulation by Ets family members. Blood 1998; 92: 3636-3646

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